Requirements of basic amino acid residues within the lectin-like domain of LOX-1 for the binding of oxidized low-density lipoprotein

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Abstract Lectin-like OxLDL receptor-1 (LOX-1) was identified as the major receptor for oxidized low-density lipoprotein (OxLDL) in aortic endothelial cells. LOX-1 is a type II membrane protein that structurally belongs to the C-type lectin family. Here, we found that the lectin-like domain of LOX-1 is essential for ligand binding, but the neck domain is not. In particular, the large loop between the third and fourth cysteine of the lectin-like domain plays a critical role for OxLDL binding as well as C-terminal end residues. Alanine-directed mutagenesis of the basic amino acid residues around this region revealed that all of the basic residues are involved in OxLDL binding. Simultaneous mutations of these basic residues almost abolished the OxLDL-binding activity of LOX-1. Electrostatic interaction between basic residues in the lectin-like domain of LOX-1 and negatively charged OxLDL is critical for the binding activity of LOX-1. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Oxidized low-density lipoprotein; Lectin-like oxidized low-density lipoprotein receptor-1; C-type lectin; Ligand-binding site

1. Introduction

Oxidized low-density lipoprotein (OxLDL) is implicated in the pathogenesis of atherosclerosis. Lectin-like OxLDL receptor-1 (LOX-1) is the receptor for atherogenic OxLDL originally identified from endothelial cells in our laboratory [1]. Recent studies have demonstrated that LOX-1 is expressed by an inducible manner not only in vascular endothelial cells, but also in monocyte-derived macrophages and smooth muscle cells [2–5]. The expression of LOX-1 is induced by inflammatory cytokines and OxLDL [6–9]. In vivo, LOX-1 expression is enhanced by some proatherogenic settings such as hypertension and hyperlipidemia, and indeed accumulated in atherosclerotic lesions [10–12].

LOX-1 is a type II membrane protein (inside out). The unique C-type lectin-like structure in the extracellular region is distinctive to other OxLDL receptors, including class A and B scavenger receptors (SR) [1]. LOX-1 is composed of four domains, an extracellular lectin-like domain at the C-terminal, connecting neck domain, transmembrane domain, and a short N-terminal cytoplasmic domain [13]. Our recent studies revealed that the lectin domain is the functional domain of LOX-1 for OxLDL binding. The conserved C-terminal residues of the lectin-like domain of LOX-1 are essential for shaping the binding pocket for both OxLDL and a neutralizing antibody for LOX-1 [14].

LOX-1-expressing cells can recognize and phagocytose a wide range of macromolecules including OxLDL as well as aged/apoptotic cells, activated platelets, and bacteria [15–17]. Since almost all of the LOX-1 ligands so far identified showed a negative charge [15,18], an ionic interaction between the ligands and LOX-1 might occur as that of the class A SR [19,20]. To identify the critical amino acids for OxLDL recognition, we generated deletion mutants and series of point mutations by replacing the basic amino acid residues with alanine. The results support that the positively charged amino acids within the lectin-like domain cooperatively recognize OxLDL.

2. Materials and methods

2.1. Lipoprotein preparations

Human LDL (1.019–1.063 g/ml) was isolated from the plasma of healthy human subjects by sequential ultracentrifugation at 4°C. Oxidative modification of LDL was performed by incubating with 7.5 μmol/l $CuSO_4$ at 37°C for 12 h. Oxidation was monitored by measuring the amount of thiobarbituric acid-reactive substances, approximately 10 nmol malondialdehyde equivalent/mg protein in OxLDL. The relative electrophoretic mobility of OxLDL to native LDL was about 2.10. Labeling of OxLDL with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes) was performed as described [1,9].

2.2. Plasmid constructs and mutagenesis

2.2.1. Truncated mutations. The wild-type and deletion mutant bovine LOX-1 cDNAs were amplified by polymerase chain reaction (PCR) with wild-type bovine LOX-1 (BLOX-1) cDNA as the template and subcloned into the pcDNA3.1/V5-His-TOPO vector (Invitrogen) that resulted in frame fusion of V5 epitope at the C-terminus [14]. The PCR primers used in this study are shown in Table 1. The overall structures of the wild-type and mutated LOX-1 proteins are schematically depicted in Fig. 2. To generate deletion of neck domain of BLOX-1, the lectin domain of BLOX-1 was amplified by PCR primers containing 5′ *Eco*RV and 3′ *Sal*I sites with wild-type BLOX-1 cDNA as the template, and these sites were used to ligate into wild-type pCDNABLOX-1-V5 that had been digested with *Eco*RV and *Xho*I. To generate Δ195–225 (between the third and fourth cysteine) mutant of BLOX-1, the fragment of 226–270 of BLOX-1 was amplified by PCR primers containing 5′ *Eco*RI and 3′ *Not*I sites with wild-type pCDNABLOX-1-V5 plasmid as the template, and these sites were used to ligate into wild-type pCDNABLOX-1-V5 that had been digested with *Eco*RI and *Not*I.

2.2.2. Site-directed mutagenesis. Specific amino acids within the lectin-like domain were substituted using the QuikChange site-di-

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rected mutagenesis kit (Stratagene, La Jolla, CA, USA) with pcDNA-BLOX-1-V5 as a template [14]. Cysteine, lysine and arginine residues were changed to alanine with mutagenic primers as shown in Table 1. Plasmids containing combination of mutations were constructed by carrying out serial mutagenesis reactions. The mutated cDNAs were sequenced completely to verify the mutations.

2.3. Transfection of mutated bovine LOX-1 cDNA into CHO cells

Wild-type CHO-K1 cells were maintained in Ham's F-12 medium (Gibco) with 10% fetal calf serum (FCS) as previously described [1]. In brief, 1.5×10^6 cells in 100-mm dishes were transfected with 20 μg plasmids using the Lipofectamine 2000 (Gibco). On the second day, cells were harvested by trypsin and replated on 12-well dishes or 2-well chamber slides. 48 h after transfections, the cells are ready for further analysis. To correct for the differences in transfection efficiencies, the cells were co-transfected with pcDNA3.1/LacZ and the LOX-1 activity was normalized with the β-galactosidase activity as described [14].

2.4. Immunoblot analysis

Cells were lysed with 62.5 mmol/l Tris–HCl (pH 7.4), 2% SDS, and 10% glycerol, equal amounts of proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore). Membranes were probed with the anti-LOX-1 monoclonal antibody (#5-2) as described [14].

2.5. Immunofluorescent staining and confocal microscopy

Binding and uptake of DiI-labeled OxLDL to LOX-1 transfected CHO cells were determined by fluorescence microscopy. Briefly, the cells were incubated with 10 μg/ml of DiI-labeled OxLDL at 37°C for 3 h. After three washes with ice-cold phosphate-buffered saline (PBS), the cells were fixed with 3.8% (v/v) paraformaldehyde in PBS for 15 min. Cells were blocked with 0.1% bovine serum albumin in PBS containing non-immune goat serum. Cells were stained with anti-V5 antibody for 60 min, and then with fluorescein isothiocyanate-conjugated goat anti-mouse IgG for 60 min in 10% FCS in PBS. The coverslips were mounted in 80% glycerol supplemented with 2% triethylenediamine and cells were examined on a confocal microscope (Fluoview, Olympus).

2.6. The binding and uptake of DiI-labeled OxLDL

Binding of DiI-OxLDL to wild-type or mutant BLOX-1 transfected CHO-K1 cells, and mock (vector) transfected CHO-K1 cells were measured after incubation in 12-well culture dishes at 4°C as previously described [1]. Ligand uptake activity was determined by the measurement of fluorescence of DiI. In brief, the transfected cells were incubated 10 μg/ml of DiI-labeled-OxLDL at 37°C for 3 h. After three washes with PBS, fluorescence microscopy was performed to detect DiI-OxLDL internalized in cells. The cells were solubilized in 0.2 ml of lysis buffer for 20 min at room temperature on shaker for protein and β-galactosidase determination. To measure the amounts of DiI-OxLDL uptake in cells, 0.3 ml of isopropanol was added to each well, and the plates were gently shaken on an orbital shaker for 15 min. The fluorescence of the extracted DiI was counted by a spectrofluorometer (Spectro Fluor, Tecan) as described [6]. Specific binding was determined by subtracting the value in the presence of a 50-fold excess of unlabeled OxLDL from the total value.

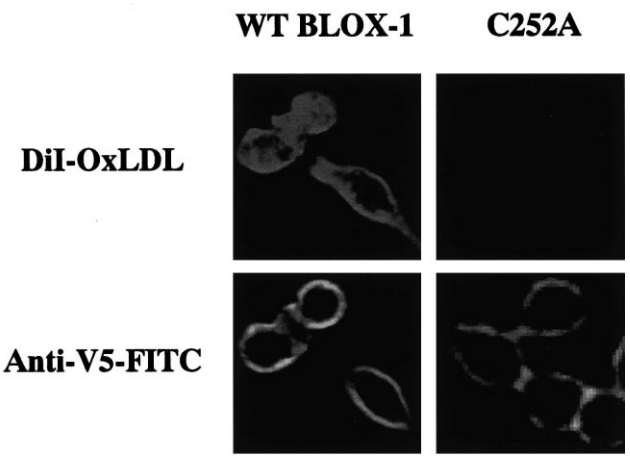

Fig. 1. Cysteine residues in the lectin domain are important to keep LOX-1's function. Point mutation in one of the conserved cysteine residues of BLOX-1, Cys252 to alanine caused the loss of OxLDL-binding activity.

3. Results and discussion

The lectin-like domain of LOX-1 was highly conserved among different species. In the previous studies, we have confirmed that lectin domain is the OxLDL-binding domain [14]. In the lectin domain, six cysteine residues are completely conserved among all species of LOX-1 proteins. According to other C-type lectin-like molecules [21,22], three disulfide bonds will be formed in bovine LOX-1, possibly bridge Cys140[Cys(I)] and Cys151[Cys(II)]; Cys168[Cys(III)] and Cys260[Cys(VI)]; Cys239[Cys(IV)] and Cys252[Cys(V)] [13]. Since mutation of the cysteine residues changes normal disulfide bond formation, the stability of LOX-1 protein basal structure might be affected. Actually, the mutation of Cys252 to alanine resulted in loss of OxLDL binding (Fig. 1). Therefore, in the present study all the cysteine residues were kept intact for the following deletion or point mutations.

In the previous study, we demonstrated the specific requirement of the nine C-terminal amino acids for the binding of OxLDL to LOX-1 with the aid of neutralizing antibody for LOX-1 [14]. To further characterize the structural requirements of LOX-1 for OxLDL binding, deletion mutants of

Table 1
Primers used for generating wild-type and mutations of BLOX-1

Construct	Sequence of the 5′ primer	Sequence of the 3′ primer
WT BLOX-1	ATGACTGTTGATGACCCCAAGG	CTGTGCTCTCAATAGATTCGCC
ΔLectin	ATGACTGTTGATGACCCCAAGG	ACCTGAATAGTTTGCTGCCTC
ΔNeck	GATATCCCTTGTCCCCAAGACTGGCTC ***Eco*RV**	GAGTCGACCGCTGTGCTCTCAATAGATTCGCC ***Sal*I**
Δ195–225	GAATTCATTCAGGGAGCTGTTTCC ***Eco*RI**	TAGAAGGCACAGTCGAGG
R205A	GGATGGGGTTGTCAATGGCGAAACCCAATTACTCGTGGC	GCCACGAGTAATTGGGTTTCGCCATTGACAACCCCATCC
K206A	GGATGGGGTTGTCAATGAGGGCACCCAATTACTCGTGGC	GCCACGAGTAATTGGGTGCCCTCATTGACAACCCCATCC
R205/K206A	GGATGGGGTTGTCAATGGCGGCACCCAATTACTCGTGGC	GCCACGAGTAATTGGGTGCCGCCATTGACAACCCCATCC
R225A	CGCCCCACTTGTTTGCAATTCAGGGAGCTGTTTCCCG	CGGGAAACAGCTCCCTGAATTGCAAACAAGTGGGGCG
R232A	CAGGGAGCTGTTTCCGCTATGTATCCTTCAGGGACC	GGTCCCTGAAGGATACATAGCGGAAACAGCTCCCTG
R244A	GGGACCTGTGCATATATTCAAGCGGGAACTGTTTTTGC	GCAAAAACAGTTCCCGCTTGAATATATGCACAGGTCCC
C252A	GTTTTTGCTGAAAACGCCATTTTAACTGCATTCAGTATATGTC	GACATATACTGAATGCAGTTAAAATGGCGTTTTCAGCAAAAAC

All sequences are from 5′ to 3′ direction. Nucleotides corresponding to restriction sites are underlined.

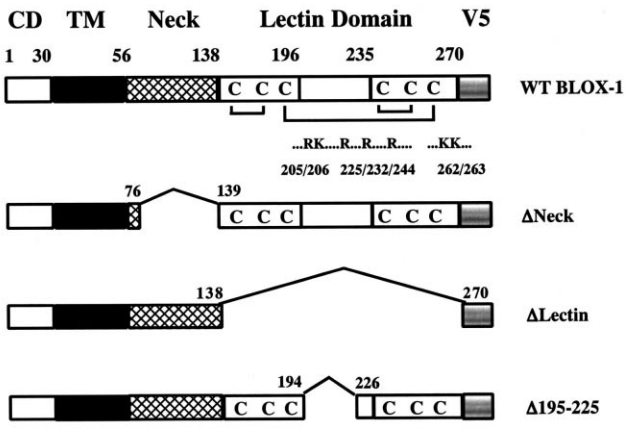

Fig. 2. Schematic illustration of BLOX-1 and its truncated and point mutant constructs. Wild-type and deletion mutant bovine LOX-1 were in frame fused to an epitope tag (V5) at the C-terminus. CD, cytoplasmic domain; TM, transmembrane domain; Neck, neck domain; Lectin domain, C-type lectin-like domain. The clusters of conserved basic amino acids residues in the lectin domain are indicated.

BLOX-1 lacking the neck domain, whole or part of the lectin-like domain were constructed (Fig. 2).

As shown in Figs. 3 and 4, deletion of the neck domain did not affect the OxLDL binding and uptake, while deletion of lectin-like domain abolished the binding activity. Therefore, the neck domain of LOX-1 does not appear to have a direct role in ligand recognition. In contrast, Δ195–225 lost OxLDL-binding activity, suggesting the importance of the structure around the deleted 31 amino acid residues in the large loop between Cys(III) and Cys(IV) in lectin-like domain. The amino acid sequence of LOX-1 has homology to NK cell receptors, including CD94, NKG2D, Ly49-A etc. [1,13]. The equivalent residues between Cys(III) and Cys(IV) of LOX-1 would be exposed to the surface according to the known crystal structure of CD94 [21,22].

It has been observed that positive electrostatic residues play significant roles in ligand–receptor interactions for SRs although the primary sequences are not necessarily identical [23,24]. The lysine clusters in the collagen domain of SR-AI/II constitute the OxLDL-binding sites [19,20]. Recently, Pearce et al reported that OxLDL binding to CD36 was sensitive to pH and ionic strength, indicating that electric interactions may be critical for binding [25]. Therefore, we sought the possibility that LOX-1 might bind OxLDL in a similar way as other SRs via the clusters of positively charged residues.

In the lectin domain three clusters of basic amino acids (at positions of R205/K206; R225/R232/R244; and K262/K263 for bovine LOX-1) are conserved among LOX-1 proteins of all known species. The importance of K262/K263 has been demonstrated in our former studies [14]. Here, we focused on the internal positively charged residues among the deleted residues in Δ195–225. A series of point mutations within this region by substituting arginine/lysine to alanine were introduced to BLOX-1.

Introduction of single point mutation to R205, K206, R225, R232 or R244 of BLOX-1 resulted in a decrease in the binding activity at 4°C, and more clearly in the binding and uptake activity at 37°C in the expressed cells, although the extents are variable (Fig. 3). Among them, mutation at R225 showed substantial effect by reducing about 50% of the binding activity, indicating a strong requirement for this residue. The effect of the mutation at R205 or K206 was minor, but showed significant effect in the combination of R205 and K206. This suggests the cooperative action of these adjoining residues. Similarly, while the single mutation at R232 or R244 showed only minor effect, the triple mutation at R225/R232/R244 decreased the binding activity more than the double mutation at R225/R232.

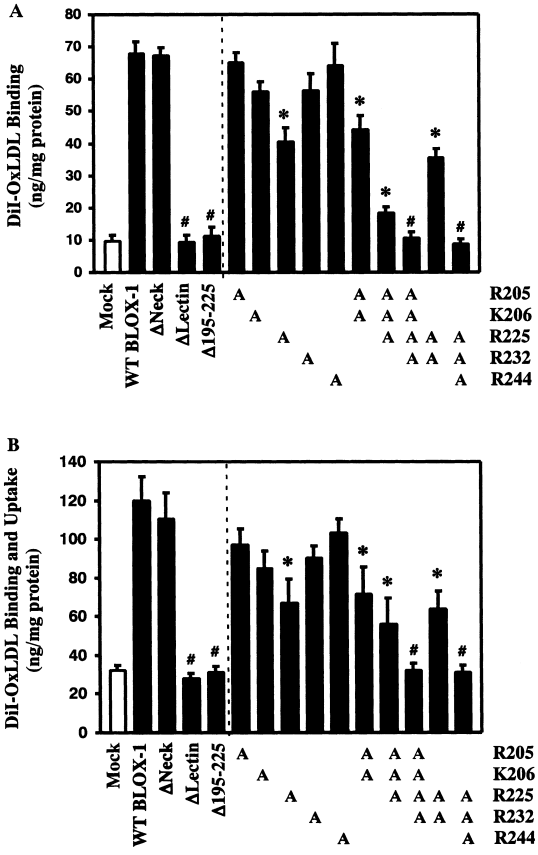

Fig. 3. OxLDL binding and uptake in CHO cells transfected with wild-type or mutant BLOX-1. A: OxLDL binding at 4°C. B: OxLDL binding and uptake at 37°C. The cells were incubated with 10 μg/ml of DiI-OxLDL for 3 h at 4°C or 37°C to examine OxLDL binding or uptake in native and mutant LOX-1 transfected CHO cells. The specific binding was calculated by subtracting the values in the presence of an excess amount of unlabeled OxLDL. Data are mean ± S.E.M. from four separate experiments. In the left side of the dashed line, the results represent wild-type and deletion mutants, and the results of point mutations are in the right. Each of the positions for substitution of the basic amino acids with alanine (A) were indicated. *, represents $P < 0.05$ vs. wild-type; #, indicates no specific binding and uptake.

Interestingly, three or more point mutations (R225/R232/R244A, R205/K206/R225/R232A) almost abolishing OxLDL binding of LOX-1 further confirmed the essential roles of the basic amino acids. The mutant LOX-1 receptors are still detected by anti-V5 antibody at the plasma membrane as well as the wild-type LOX-1, indicating the cell surface sorting of LOX-1 was not affected (Fig. 4). In addition, the expression of mutant LOX-1 in correct size can be detected by Western blotting with anti-LOX-1 monoclonal antibody (Fig. 5). Therefore, the loss of OxLDL-binding activity in mutant LOX-1-expressing cells should be ascribed to the impairment of ligand–receptor interaction.

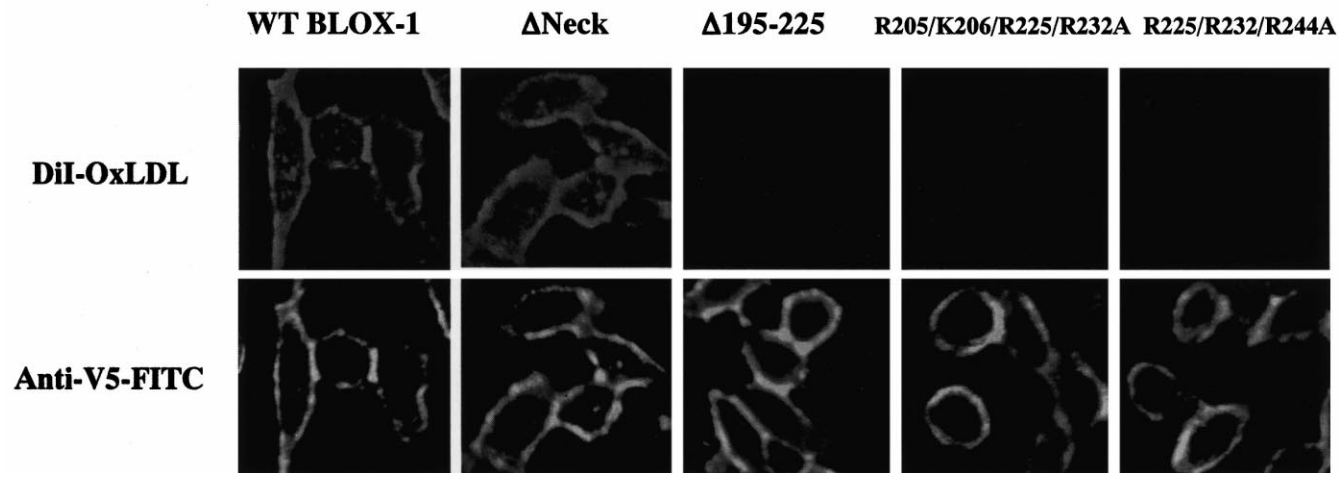

Fig. 4. Confocal fluorescence microscopy of transfected CHO cells with wild-type and mutations of BLOX-1. The top images showed the activity of binding and uptake of DiI-OxLDL. The bottom images illustrated the staining with anti-V5 antibody. Deletion of Neck domain of LOX-1 still keeps the activity of binding and uptake of OxLDL as well as wild-type. While deletion of 31 amino acids (Δ195–225) of lectin domain or multiple point mutations of the basic amino acids, R205/K206/R225/R232A or R225/R232/R244A failed to bind OxLDL. Both of the wild-type and mutant BLOX-1 were essentially stained in the cell membrane by anti-V5 antibody.

The simplest explanation of the results is that the positively charged residues might directly serve as binding pocket. OxLDL exhibits strong negative charge since the lipid peroxidation products were generated and linked to the degraded ApoB moiety [26]. An ionic interaction between LOX-1 receptor and ligand would, therefore, occur at physiologic pH. It is possible that LOX-1 ligands interact directly with those of the charged residues within the lectin-like domain. The mutations on basic amino acid residues may impair the interaction in the ligand-binding pocket.

Alternatively, the lectin-like domain must adopt a precise conformation to achieve ligand binding, possibly through intramolecular ionic interaction. The charged residues in the lectin domain are critical to stabilize the delicate structure. It is possible that mutations on the charged residues will alter the delicate conformations of the receptor as well.

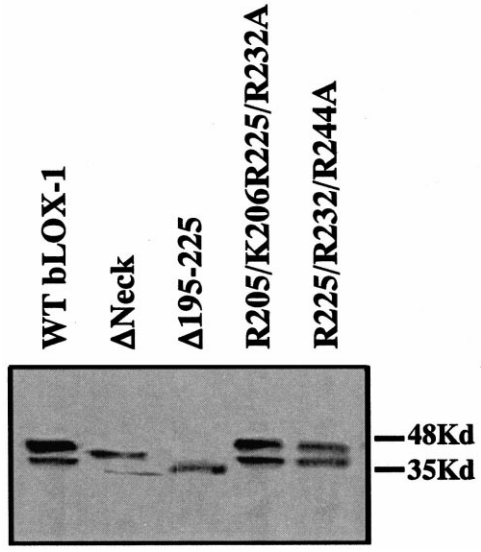

Fig. 5. Immunoblot analysis confirmed mutant BLOX-1 proteins expressed in CHO cells. CHO cells were transfected by either wild-type or mutant BLOX-1 expression vector. LOX-1 proteins were detected by a monoclonal antibody for BLOX-1.

The identification of the amino acid residues that is essential for OxLDL binding will give a clue to design the antagonists for LOX-1.

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