Conserved C-terminal residues within the lectin-like domain of LOX-1 are essential for oxidized low-density-lipoprotein binding

Mingyi CHEN*†, Shuh NARUMIYA†, Tomoh MASAKI* and Tatsuya SAWAMURA*‡[1]

*National Cardiovascular Center Research Institute, Suita, Osaka 565-8565, Japan, †Department of Pharmacology, Faculty of Medicine, Kyoto University, Kyoto 606, Japan, and ‡Department of Molecular Pathophysiology, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan

Lectin-like oxidized low-density-lipoprotein (oxLDL) receptor-1 (LOX-1) is a cell-surface endocytosis receptor for atherogenic oxLDL, which is highly expressed in endothelial cells. Recent studies suggest that it may play significant roles in atherogenesis. LOX-1 is a type-II membrane protein that structurally belongs to the C-type lectin family molecules. This study was designed to characterize the specific domain on LOX-1 that recognizes oxLDL. Truncation of the lectin domain of LOX-1 abrogated oxLDL-binding activity. Deletion of the utmost C-terminal ten amino acid residues (261–270) was enough to disrupt the oxLDL-binding activity. Substitutions of Lys-262 and/or Lys-263 with Ala additively attenuated the activity. Serial-deletion analysis showed that residues up to 265 are required for the expression of minimal binding activity, although deletion of the C-terminal three residues (268–270) still retained full binding activity. Consistently, these alterations in LOX-1 impaired the recognition by a functionally blocking monoclonal antibody for LOX-1. These data demonstrated the distinct role of the lectin domain as the functional domain recognizing LOX-1 ligand. The conserved C-terminal residues of lectin-like domain are essential for binding oxLDL. Particularly, the basic amino acid pair is important for the binding.

Key words: epitope mapping, lectin-like oxidized LDL receptor-1, monoclonal antibody.

INTRODUCTION

Lectin-like oxidized low-density-lipoprotein (oxLDL) receptor-1 (LOX-1) was initially identified in vascular endothelial cells as a cell-surface endocytosis receptor for oxLDL [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 many pro-inflammatory cytokines and oxLDL [6–9]. *In vivo*, LOX-1 expression is enhanced by some pro-atherogenic settings such as hypertension and hyperlipidaemia, and indeed in atherosclerotic lesions [10–12]. Further evidence has accumulated about the pathophysiological consequences of oxLDL binding to LOX-1, including activation of nuclear factor κB through an increased production of reactive oxygen species [13], subsequently inducing monocyte adhesion to endothelial cells [14] and endothelial apoptosis [15]. All of these findings support the relevance of this receptor to atherogenesis. Moreover, LOX-1-expressing cells can also recognize and phagocytose activated platelets, as well as aged/apoptotic cells, suggesting its versatile physiological functions [16,17].

LOX-1 is a 50 kDa type-II membrane protein that structurally belongs to the C-type lectin family. Interestingly, LOX-1 does not share any structural homology with other known receptors for oxLDL, including class-A and -B scavenger receptors [1,18,19]. LOX-1 is composed of four functional domains, short N-terminal cytoplasmic domain, transmembrane domain, connecting neck domain and lectin-like domain at the C-terminal [20]. Notably, the lectin-like domain is highly conserved among species, especially at the positions of the six cysteine residues. This suggests that the lectin-like domain might be a recognition domain for oxLDL, consequently initiating the processes of internalization and phagocytosis [11]. The present study was undertaken to characterize the structure–function relationship of LOX-1. Serial-deletion and site-directed mutagenesis of the lectin domain were performed. All the evidence collected supports the involvement of the specific region of the lectin-like domain in recognition and binding of oxLDL.

EXPERIMENTAL PROCEDURES

cDNA cloning of porcine LOX-1 and sequence analysis

Porcine thoracic aorta was obtained from a white pig in a local slaughter house. Porcine aortic endothelial cells were harvested, and a cDNA library was constructed with size-fractionated cDNA (> 500 bp) in λgt10 as described in [11]. Approx. 5×10^5 clones were screened at high-stringency hybridization conditions using the coding region of human LOX-1 cDNA as a probe. Three positive clones containing a > 1.5 kb insert with the full open reading frame were identified. The inserts were subcloned to pUC18 vector for sequencing. The nucleotide sequences were determined on both strands using the dideoxynucleotide chain-termination method with a LI-COR DNA sequencer (model

Abbreviations used: oxLDL, oxidized low-density lipoprotein; LOX-1, lectin-like oxLDL receptor-1; bLOX-1, bovine LOX-1; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; CHO, Chinese hamster ovary; FCS, fetal calf serum; SR, scavenger receptor.

[1] To whom correspondence should be addressed, at the National Cardiovascular Center Research Institute (e-mail sawamura@ri.ncvc.go.jp).

The nucleotide sequence data reported for porcine LOX-1 will appear in the GenBank® Nucleotide Sequence Database under the accession number AB018668.

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4000L). Nucleotide and amino acid sequences were analysed and compared with other species' sequences using Gene Works software (Intelligenetics) on a Macintosh computer.

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 μM $CuSO_4$ at 37 °C for 12 h. Oxidation was monitored by measuring the amount of thiobarbituric acid-reactive substances, approx. 10 nmol of malondialdehyde equivalent/mg of protein in oxLDL. The relative electrophoretic mobility of oxLDL to native LDL was about 2.10. Labelling of oxLDL with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes) was performed as described in [1,21].

Cell culture

All cell-culture incubations were at 37 °C in 95 % air/5 % CO_2. Wild-type Chinese hamster ovary (CHO)-K1 cells were maintained in Ham's F-12 medium (Gibco) with 10 % fetal calf serum (FCS). CHO-K1 cells stably expressing bovine LOX-1 (bLOX-1-CHO) were maintained in Ham's F-12/10 % FCS supplemented with 10 μg/ml blasticidin S as described previously [1,16,21].

Plasmid constructs and mutagenesis

Truncated mutations

The wild-type and mutant bLOX-1 cDNA with stop codon were amplified by PCR and subcloned into the PCR3.1 vector (Invitrogen). Accordingly, the PCR fragments generated by reverse primer without stop codon were subcloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen), resulting in the in-frame fusion of the V5 epitope at the C-terminus. The PCR primers used in this study were shown in Table 1. The overall structures of the wild-type and mutated LOX-1 proteins are depicted schematically in Figures 2 and 6 (see below).

Site-directed mutagenesis

Specific amino acids within the lectin-like domain were substituted using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). Briefly, using bLOX-1 cDNA in PCR3.1 mammalian expression vector as template, two complementary primers (125 ng each) containing the desired mutation and 50 ng of template in 1 × reaction buffer were denatured at 95 °C for 30 s and annealed at 55 °C for 1 min, and DNA synthesis was carried out by *Pfu* polymerase at 68 °C for 14 min; this cycle was repeated 18 times. The methylated template was removed by incubation with 10 units of *Dpn*I at 37 °C for 1 h. Three types of alanine substitution were constructed; Lys262 and/or Lys263 were changed to alanine with mutagenic primers as shown in Table 1. Plasmids containing a combination of mutations were constructed by carrying out serial mutagenesis reactions. The mutated cDNAs were sequenced completely to verify the mutations. The sequencing reactions were performed with ABI Dye Terminator kit and analysed on ABI model 310 sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

Transfection of wild-type and mutated bLOX-1 cDNA into CHO cells

Wild-type CHO-K1 cells were maintained in Ham's F-12 medium with 10 % FCS. In brief, 1.5 × 10^6 of cells in 100 mm dishes were

Table 1 Primers used in the construction of wild-type and mutant bLOX-1 by PCR

Primer	Sequence (5′ → 3′)
Forward primer	
bLOX-1	ATG ACT GTT GAT GAC CCC AAG G
Reverse primers for generating V5-epitope-tagged fusion proteins	
bLOX-1	CTG TGC TCT CAA TAG ATT CGC C
Δ261	GCA TAT ACT GAA TGC AGT TAA AAT GC
Δ257	TGC AGT TAA AAT GCA GTT TTC AGC
Δ173	CAA AGA CAA GCA GTT CTC CTG
Δ158	GCC AGA GGA AAA TTG GTA ACA G
Δ143	TTG GGG ACA AGG ACC TGA ATA G
ΔLectin	ACC TGA ATA GTT TGC TGC CTC
Reverse primers with stop codon	
bLOX-1	CTA CTG TGC TCT CAA TAG ATT CGC CTT C
Δ268	CTA TAG ATT CGC CTT CTT TTG ACA TAT ACT GAA
Δ266	CTA ATT CGC CTT CTT TTG ACA TAT ACT G
Δ265A	CTA AGC CGC CTT CTT TTG ACA TAT ACT G
Δ265D	CTA ATC CGC CTT CTT TTG ACA TAT ACT G
Δ265	CTA CGC CTT CTT TTG ACA TAT ACT GAA TGC
Δ264	CTA CTT CTT TTG ACA TAT ACT GAA TGC AG
Δ263	CTA CTT TTG ACA TAT ACT GAA TGC AGT TAA AAT GC
Δ262	CTA TTG ACA TAT ACT GAA TGC AGT TAA AAT GC
Primers for site-directed mutagenesis of bLOX-1	
K262Af	AGT ATA TGT CAA GTG AAG GCG AAT CTA TTG AGA GCA CAG
K262Ar	CTG TGC TCT CAA TAG ATT CGC CTT CAC TTG ACA TAT ACT
K263Af	AGT ATA TGT CAA AAG GCG GCG AAT CTA TTG AGA GCA CAG
K263Ar	CTG TGC TCT CAA TAG ATT CGC CGC CTT TTG ACA TAT ACT
K262/263Af	AGT ATA TGT CAA GCA GCG GCG AAT CTA TTG AGA GCA CAG
K262/263Ar	CTG TGC TCT CAA TAG ATT CGC CGC TGC TTG ACA TAT ACT

transfected with 20 μg of plasmids using Lipofectamine 2000 (Gibco). On the second day, cells were harvested by trypsin and replated on 12-well dishes or 2-well chamber slides. After transfections (48 h), 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.

Immunofluorescent staining and microscopy

Binding and uptake of DiI-labelled oxLDL to LOX-1-transfected CHO cells were determined by fluorescence microscopy. Briefly, the cells were incubated with 10 μg/ml DiI-labelled oxLDL at 4 or 37 °C for 3 h. After washing three times with ice-cold PBS, the cells were fixed with 3.8 % (v/v) paraformaldehyde in PBS for 15 min. Cells were blocked with 0.1 % BSA in PBS containing non-immune goat serum. Cells were stained for LOX-1 or V5 epitope by incubating with an anti-LOX-1 or anti-V5 antibody for 60 min, and then with FITC-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 Zeiss Axiovert microscope. Fluorescent images were recorded on Kodak Ektachrome film.

Generation of anti-bLOX-1 monoclonal antibodies

Anti-bLOX-1 monoclonal antibodies were generated by immunizing Balb/c mice with bLOX-1-CHO cells. Hybridoma from the splenocytes was prepared by standard procedures, and screened by cell-surface immunobinding to bLOX-1-CHO cells [9,22]. A functional blocking antibody (JTX-20) was further

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bovine    MTV----DDP K--GMKDQLD QKPNGKTAKG F--VSSWRWY PAAVTLGVLC    42
human     MTF----DDL KIQTVKDQPD EKSNGKKAKG LQFLYSPWWC LAAATLGVLC    46
porcine   MTL----DDL KSNSMKDQPD EKSNGDKAEG PRSLSTLRWR PAALILGLLC    46
rabbit    MNLEMAVDDL KVKPMKDQPD QKSNGKKPKG LRFLSSPWWC PAAVALGVLC    50
rat       MAF----DD- KMKPVNGQPD QKSCGKKPKG LHLLSSTWWC PAAVTLAILC    45
mouse     MTF----DD- KMKPANDEPD QKSCGKKPKG LHLLSSPWWF PAAMTLVILC    45

              Cytoplasmic domain

bovine    LGLLVTVILL ILQLSQVSDL IKKQQANITH QEDILEGQIL AQRRSEKSAQ    92
human     LGLVVTIMVL GMQLSQVSDL LTQEQANLTH QKKKLEGQIS ARQQAEEASQ    96
porcine   LGLLVTVILL IIQLSQVSDL LKQQKVKLTH QEDILEGQAL AQRQAEKSSQ    96
rabbit    LGSLMTIIML GMQLLQVSDL LKQQQANLTL QENILEGQVL AQQQAEAASQ   100
rat       LVLSVTLIVQ QTQLLQVSDL LKQYQANLTQ QDHILEGQMS AQKKAENASQ    95
mouse     LVLSVTLIVQ WTQLRQVSDL LKQYQANLTQ QDRILEGQML AQQKAENTSQ    95

          TMD

bovine    ---------- ---------- ---------- ---------- ----------    92
human     ---------- ---------- ---------- ---------- ----------    96
porcine   ---------- ---------- ---------- ---------- ----------    96
rabbit    ---------- ---------- ---------- ---------- ----------   100
rat       ESKRELKEQI DTLTWKLNEK SKEQEKLLQQ NQNLQEALQR AVNASEESKW   145
mouse     ESKKELKGKI DTLTQKLNEK SKEQEELLQK NQNLQEALQR AANSSEESQR   145

                        Neck Domain

bovine    ---------- ---------- ---------- ---------- --ESQKELKE   100
human     ---------- ---------- ---------- ---------- --ESENELKE   104
porcine   ---------- ---------- ---------- ---------- --ESQRELTE   104
rabbit    ---------- ---------- ---------- ---------- --ESQRELKE   108
rat       ELKEQIDILN WKLNGISKEQ KELLQQNQNL QEALQKAEKY SEESQRELKE   195
mouse     ELKGKIDTIT RKLDEKSKEQ EELLQMIQNL QEALQRAANS SEESQRELKG   195

bovine    MIETLAHKLD EKSKKLMELH RQNLNLQEVL KEAANYSGPC PQDWLWHEEN   150
human     MIETLARKLN EKSKEQMELH HQNLNLQETL KRVANCSAPC PQDWIWHGEN   154
porcine   MIETLAHKLD EKSKKLMELQ QQNLNLQKAL EKAANFSGPC PQDWLWHEEN   154
rabbit    MIETLAKRLD EKSKKQMELN HQYLNLQEAL KRMDNFSGPC PEDWLWHGKN   158
rat       QIDTLSWKLN EKSKEQEELL QQNQNLQEAL QRAANSSGPC PQDWIWHKEN   245
mouse     KIDTLTLKLN EKSKEQEELL QKNQNLQEAL QRAANFSGPC PQDWLWHKEN   245
                                                 *

bovine    CYQFSSGSFN WEKSQENCLS LDAHLLKINS TDELEFIQQM IAHSSFPFWM   200
human     CYLFSSGSFN WEKSQEKCLS LDAKLLKINS TADLDFIQQA ISYSSFPFWM   204
porcine   CYKFSSGPFS WEKSRENCLS LDAQLLKINS TDDLEFIQQT IAHSSFPFWM   204
rabbit    CYLFSSGSFN WESSQEKCLS LDAQLLKINS TEDLGFIQQA TSHSSFPFWM   208
rat       CYLF-HGPFN WEKSRENCLS LDAQLLQIST TDDLNFVLQA TSHSTSPFWM   294
mouse     CYLF-HGPFS WEKNRQTCQS LGGQLLQING ADDLTFILQA ISHTTSPFWI   294
          *                  *  C-type Lectin Domain

bovine    GLSMRKPNYS WLWEDGTPLT PHLFRIQGAV SRMYPSGTCA YIQRGTVFAE   250
human     GLSRRNPSYP WLWEDGSPLM PHLFRVRGAV SQTYPSGTCA YIQRGAVYAE   254
porcine   GLSLRKPNNS WLWEDGTPLM PHLFRLQGAA SQMYPSGTCA YIHRGIVFAE   254
rabbit    GLSRRKPDYS WLWEDGSPLM PHLFRFQGAV SQRYPSGTCA YIQKGNVFAE   258
rat       GLHRKNPNHP WLWENGSPLS FQFFRTRGVS LQMYSSGTCA YIQGGVVFAE   344
mouse     GLHRKKPGQP WLWENGTPLN FQFFKTRGVS LQLYSSGNCA YLQDGAVFAE   344
                                               *

bovine    NCILTAFSIC QKKANLLRAQ                                    270
human     NCILAAFSIC QKKANL-RAQ                                    273
porcine   NCILNAFSIC QKRANLLRAQ                                    274
rabbit    NCILVAYSIC QKKANLLRSE                                    278
rat       NCILTAFSIC QKKANLLLTQ                                    364
mouse     NCILIAFSIC QKKTNHLQI-                                    363
            *      *
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Figure 1 Comparison of the amino acid sequences of LOX-1

Alignment of the deduced amino acid sequences of bovine, human, porcine, rabbit, rat and mouse LOX-1. The putative transmembrane domain (TMD) is indicated by a solid underline, and the C-type lectin domain is indicated by a dashed underline. Asterisks indicate the conserved sic cysteine residues. The shaded box highlights the consensus C-terminal residues essential for oxLDL binding. The bold letters represent the conserved basic amino acids in the putative ligand-binding domain.

selected for blocking DiI-labelled oxLDL binding and uptake in bLOX-1-expressing cells as described in [5,13].

Immunoblot analysis

Cells were lysed with 62.5 mM Tris/HCl (pH 7.4), 2 % SDS and 10 % glycerol, and protein concentrations were determined by using a BCA protein assay kit (Pierce Chemical) with BSA as standard. Samples containing 60 μg of protein were separated by SDS/PAGE and transferred on to PVDF membrane (Immobilon, Millipore). Membranes were probed with the anti-LOX-1 monoclonal antibodies 5-2, JTX-20 and anti-V5 antibody followed by horseradish peroxidase-conjugated horse anti-mouse

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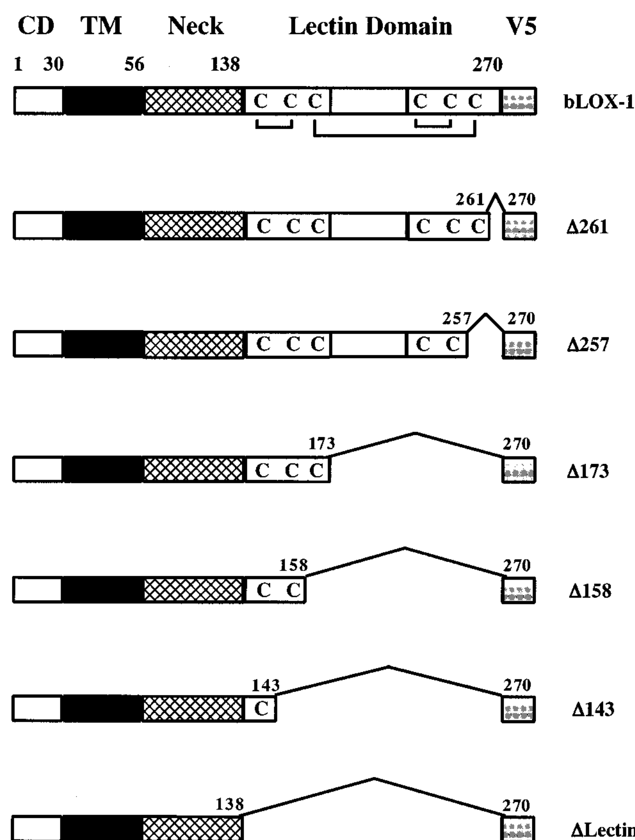

Figure 2 Schematic diagram of the wild-type and truncation constructs of LOX-1

Wild-type and deletion mutants of bLOX-1 were fused in-frame to an epitope tag (V5) at the C-terminus. CD, cytoplasmic domain; TM, transmembrane domain; Neck, neck domain; Lectin, C-type lectin-like domain.

antibody. Bands were visualized by Vectastain Elite ABC kit (Vector) as described in [11].

The binding and uptake of DiI-labelled oxLDL

Binding of DiI-labelled 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 described previously [1,6,21]. Ligand-binding and -uptake activity was measured by fluorescently labelled oxLDL uptake. In brief, the transfected cells were incubated with 10 μg/ml DiI-labelled oxLDL at 37 °C for 3 h. After three washes with PBS, fluorescence microscopy was performed to detect the DiI-labelled oxLDL internalized in cells. The cells were solubilized in 0.2 ml of lysis buffer for 20 min at room temperature on a rotary shaker for protein and β-galactosidase determination. To measure the amounts of DiI-labelled oxLDL uptake in cells, 0.3 ml of isopropanol was added to each well, and the plates were shaken gently on an orbital shaker for 15 min. The fluorescence of the extracted DiI was counted by a spectrofluorometer (Spectro Fluor, Tecan) as described [6]. Non-specific binding was determined in the presence of a 50-fold excess of unlabelled oxLDL. The specific binding activities were defined by subtracting non-specific values from the total binding activities.

RESULTS

Structural analysis of LOX-1 among different species

LOX-1 was initially cloned from bovine aortic endothelial cells. So far, we have identified the human, rat, mouse and rabbit LOX-1 homologues [1,10,11,23]. In this study, we also cloned porcine LOX-1 cDNA. The sequence spanned 1578 nucleotides, and comprises a 5′-untranslated region of 44 nucleotides, an open reading frame of 822 nucleotides and a 3′-untranslated region of 709 nucleotides. The open reading frame encoded a protein comprising 274 amino acids with 69 % homology to human LOX-1. Alignment of the deduced amino acid sequences revealed a high degree of conservation in the lectin-like domains of LOX-1 proteins. In particular, the positions of six cysteine residues are completely conserved among all the known species. LOX-1 structurally belongs to the lectin-like receptors, which are characterized as type-II membrane-integrated proteins containing a single carbohydrate-recognition domain (CRD) motif in the C-terminus. The lectin-like domain is likely to be the ligand-binding domain, as is the case for other lectin-like receptors. At the C-terminal end of LOX-1, at least six amino acids between position 260 and 265 were well conserved among the species tested (Figure 1). Notably, a pair of basic amino acids is present in all species, exhibited as Lys-262/Lys-263 in bLOX-1, whereas instead the equivalent amino acids are Lys and Arg in porcine LOX-1. Residues conserved across species for LOX-1 are likely to be critical for its function.

Lectin-like domain is the ligand-binding domain of LOX-1

Targeted mutations were made in the extracellular domain to identify structure required for LOX-1's function. Truncation of the lectin domain of LOX-1 (ΔLectin, see Table 1) results in loss of oxLDL binding (Figures 2 and 3). Furthermore, we found that even when the last ten amino acids were deleted (Δ261) the mutant receptor failed to bind oxLDL. In addition, when lacking the lectin domain, LOX-1 loses the function of recognition and binding of aged/apoptotic cells and negatively charged phospholipids (results not shown). These observations further support the notion that the unique lectin-like structure mediates LOX-1's recognition of its ligand. The cell-surface expression of mutant forms of LOX-1 were confirmed by staining with anti-V5 monoclonal antibody without permeabilization of plasma membrane (Figure 3). Therefore, the loss of the binding to ligands is not due to interference of the sorting of LOX-1 to plasma membrane.

Mapping of epitopes recognized by monoclonal antibodies

The strategy for generating a serial-deletion mutant of bLOX-1 and the in-frame fusion of the V5 epitope to the C-terminus is indicated in Figure 2. For the fine mapping of the ligand-binding domain of LOX-1, we utilized two kinds of monoclonal antibody, 5-2 and JTX-20. The latter is a functional blocking antibody (Figure 4). bLOX-1 was subjected to serial-deletion mutagenesis and Western-blotting analyses with these monoclonal antibodies (Figure 5). Mapping of epitopes showed that the monoclonal antibody 5-2 bound a motif within residues 158–172 between the second and third cysteine residues. The neutralizing antibody, JTX-20, which recognizes LOX-1, requires the integrity of C-terminal residues 261–270, which are also required for the binding of oxLDL, as discussed above.

Analysis of the C-terminal ten residues

To determine the role of the C-terminal ten residues in oxLDL binding, we have constructed serial-truncation mutations

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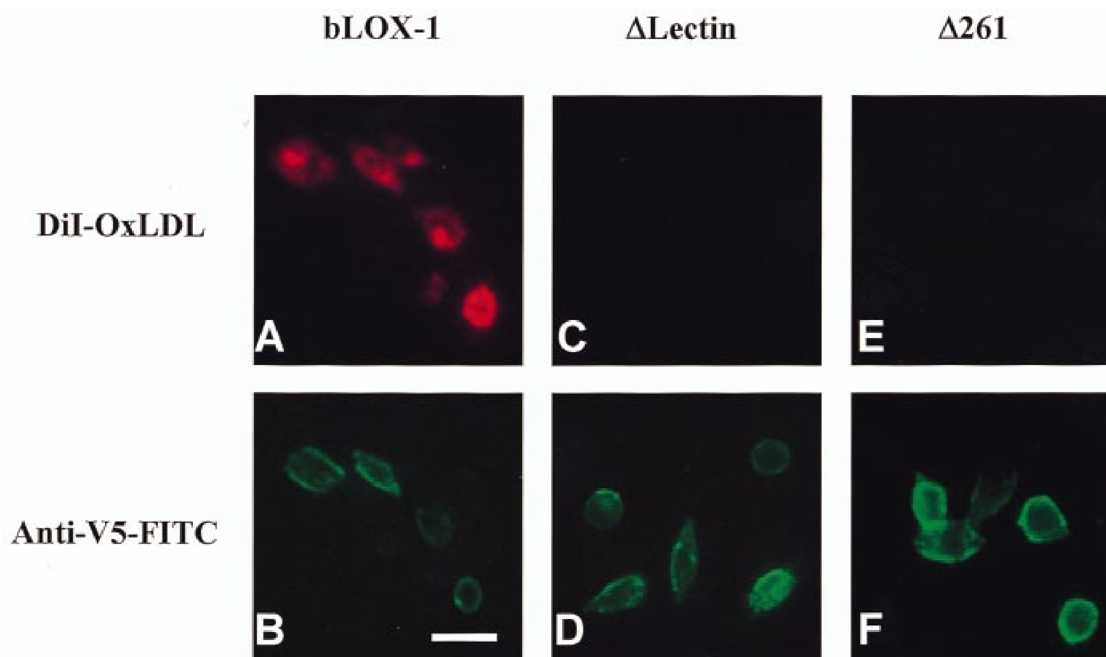

Figure 3 Fluorescence microscopy of CHO cells transiently transfected with wild-type and deletion mutants of bLOX-1

CHO cells were transiently transfected with bLOX-1 (**A** and **B**), ΔLectin (**C** and **D**) and Δ261 (**E** and **F**), shown in Figure 2. After transfection (2 days), the cells were incubated with 10 μg/ml of DiI-labelled oxLDL for 3 h at 37 °C. After fixation without permeabilization, the cells were stained with FITC-labelled anti-V5 monoclonal antibody and examined with a fluorescence microscope (Zeiss). The upper panels show the activity of binding and uptake of DiI-labelled oxLDL. The lower panels indicate the expression of LOX-1 receptor by staining with anti-V5 antibody. Scale bar, 10 μm. Deletion mutants of LOX-1 lacking the whole lectin-like domain or C-terminal residues 261–270 failed to bind oxLDL (**C** and **E**).

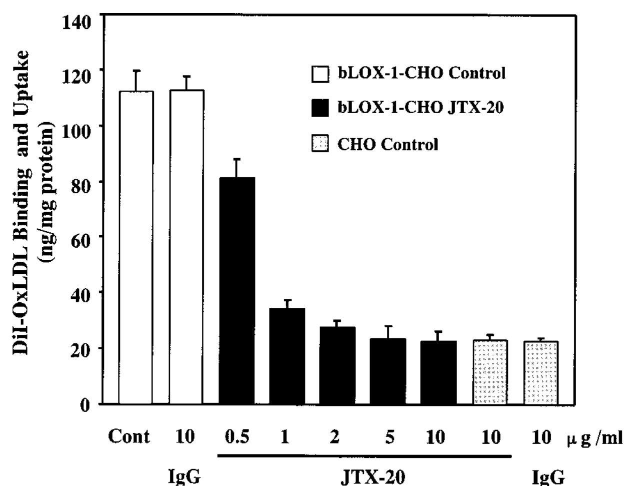

Figure 4 Characterization of a neutralizing antibody for bLOX-1

A mice anti-LOX-1 monoclonal antibody (JTX-20) was applied in this study. bLOX-1-CHO and wild-type CHO cells were incubated with 10 μg/ml DiI-labelled oxLDL for 3 h at 37 °C in the presence of JTX-20 or normal mice IgG at concentrations as indicated. The cell binding and uptake of DiI-labelled oxLDL was measured as described in the Experimental procedures section. Values represent the means ± S.D. from three separate experiments. JTX-20 was characterized as a functional blocking antibody for bLOX-1.

affecting amino acids 261–270 (Figure 6). Since all the cysteine residues are still conserved, the basal conformation of mutant LOX-1 is likely to remain intact as a native receptor. As shown in Figure 7, deletion of 268–270 (Δ268) did not significantly alter the ligand-binding activity. Truncation Leu-266 (Δ266) partially reduced the binding, whereas truncation at Asn-265 (Δ265) totally disrupted the oxLDL-binding activity. The Asn-265 → Ala mutant of Δ266 (Δ265A) bound oxLDL as much as Δ266, whereas the Asn-265 → Asp mutant of Δ266 (Δ265D) showed decreased activity. Much shorter truncations (Δ262–Δ264) did not show specific oxLDL-binding/-uptake activity. These results suggest that C-terminal length up to 265 is essential and that residues up to 267 are sufficient for ligand-binding activity.

We then focused on the basic amino acid pair located at 262–263. Substitutions of Lys-262 or Lys-263 to alanine (K262A, K263A) moderately reduced the binding and internalization of oxLDL. When both lysine residues were replaced simultaneously (K262/263A), the ligand binding was decreased further. This indicates that positive charge of the pair of basic amino acid residues constitutes an important part of the ligand-binding activity.

Interestingly, Western-blot analysis demonstrated that the integrity of amino acids spanning 261–265 is required for recognition by the neutralizing antibody, JTX-20. The mutations affecting ligand-binding activity impaired the recognition by JTX-20 in parallel (Figure 7, bottom panel). By Western blotting, two bands of the LOX-1 protein were detected. This is ascribed to variation in the post-translational modifications by N-linked glycosylation. Although we have reported that the change in N-glycosylation of LOX-1 affect the binding of oxLDL [22], the C-terminal mutations after Cys-260 do not alter the signals for the N-glycosylation site. In fact, all the mutants showed two bands in a similar pattern by Western blotting with antibody 5-2. Therefore, changes in the binding of oxLDL in the mutants should be ascribed to changes in amino acid residues, but not in the carbohydrate.

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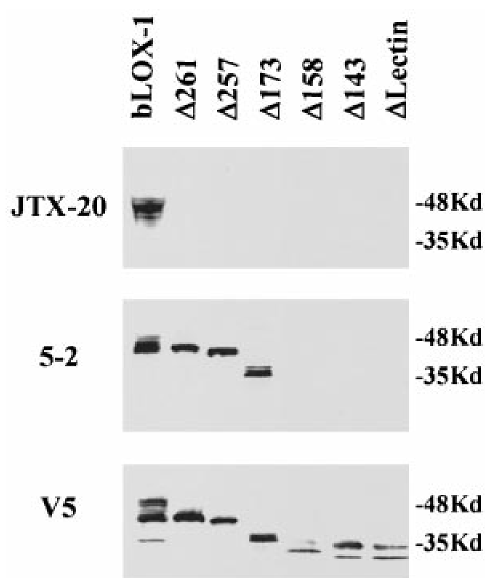

Figure 5 Immunoblot analysis of wild-type and serial-deletion mutants of the lectin-like domain of bLOX-1

Wild-type and serial-deletion mutants of LOX-1 (shown in Figure 2) transfected into CHO cells were analysed by Western blot to characterize the epitopes. The 5-2-binding motif was localized between amino acids 158 and 172 of bLOX-1. The C-terminal residues between amino acids 261 and 270 are required for recognition by JTX-20.

		249 … 260-1 -2 -3 -4 -5 … 270
bLOX-1	:	**---A E N C I L T A S I C Q K K A N L L R A Q**
K262A	:	**-----------------------------------A------------------------**
K263A	:	**--------------------------------------A---------------------**
K262/263A	:	**-----------------------------------A A--------------------**
Δ268	:	**-----------------------------C Q K K A N L L**
Δ266	:	**-----------------------------C Q K K A N**
Δ265A	:	**-----------------------------C Q K K A A**
Δ265D	:	**-----------------------------C Q K K A D**
Δ265	:	**-----------------------------C Q K K A**
Δ264	:	**-----------------------------C Q K K**
Δ263	:	**-----------------------------C Q K**
Δ262	:	**-----------------------------C Q**

Figure 6 The amino acid sequences of the mutants in the C-terminal of lectin-like domain of bLOX-1

The dashed lines represent unchanged amino acids from the native bLOX-1. Underlined characters show the amino acid residues of the LOX-1 protein that are conserved between species.

DISCUSSION

We initially identified LOX-1 from aortic endothelial cells. It is a cell-surface endocytosis receptor for oxLDL [1]. LOX-1 showed relatively lower efficiency in uptake of oxLDL *in vitro* in the present study than scavenger receptor SR-AI. Doi et al. [24] reported that oxLDL binding to bovine-SR-AI-transfected COS cells was about 2.7 ng/mg of protein at 4 °C and 6.0 ng/mg of protein at 37 °C. In the present study, the values were 67 ng/mg of protein at 4 °C, and 109 ng/mg of protein at 37 °C for bovine LOX-1-transfected CHO cells. This might be reflecting the expression profiles of the receptors. SR-A is mainly expressed in macrophages, the professional phagocytes. LOX-1 is mainly expressed in endothelial cells, non-professional phagocytes. As an endothelial receptor for oxLDL, the major role of LOX-1 may be to induce signal transduction and gene expression leading to the functional change of endothelial cells [13,14]. However, the relative inefficiency of the uptake of oxLDL may indicate that this might be a rate-limiting process for the accumulation of lipoprotein in blood vessels. Although the mechanisms for the accumulation of lipoproteins in arterial wall have not been fully clarified, we have found that LOX-1 plays a significant role at least in the distribution of exogenously administrated oxLDL (results not shown).

The unique lectin-like structure of LOX-1 is distinct from other oxLDL receptors. Therefore, to characterize its structure–function relationship is of critical significance [11,12]. The lectin-like domain was formerly predicted as the ligand-binding domain [11]. In this study, the deletion analysis confirms that a specific region of the lectin-like domain is essential for binding and endocytosis of LOX-1 ligand. Notably, even deletion of the C-terminal ten amino acids of the lectin-like domain can completely disrupt the oxLDL binding. In this region, at least six amino acid residues including two basic amino acids were completely conserved among different species of LOX-1. We further utilized serial-deletion and point mutations of amino acids 261–270 at the C-terminal end of the lectin-like domain to identify the structural motif of LOX-1 that recognizes oxLDL. Since the six cysteine residues were kept intact in all these mutations, the basal conformation of LOX-1 should be kept unchanged. This was supported by the correct sorting of the mutant receptors to the plasma membrane (Figure 3). In the absence of amino acids 268–270, the activity of oxLDL binding was essentially unchanged, whereas deletion of amino acid 265–270 abolishes the binding. Interestingly, almost all of the lectin-like receptors have at least four amino acid residues succeeding the last cysteine residue. The length of C-terminal residues may be significant for ligand binding in common among the lectin-like receptors. On the other hand, the substitutions of Lys-262 → Ala, Lys-263 → Ala and Asn-265 → Asp, which decrease positive charge, greatly attenuate the binding of oxLDL. Collectively, the positive charge and the length of the utmost C-terminal of lectin domain are critical for the ligand binding of LOX-1. We suggest that the six amino acid residues (260–265) constitute at least one of the ligand-binding sites of bLOX-1.

Since the present study was performed with a submaximal dose of oxLDL, the reduced binding of oxLDL suggests a change in affinity. It is likely that a charge-dependent mechanism affected the affinity of LOX-1 to oxLDL at the distal segment of the lectin-like domain. The conserved Lys/Arg residues at the C-terminal of the lectin-like domain might contribute to the stabilization of the binding complex by charge interactions. The positively charged Arg and Lys residues of the apolipoprotein B-100 moiety of LDL are known to play a key role in recognition by classic LDL receptor. During oxidative modification of LDL, the lipid peroxidation results in the generation of aldehydes that substitute lysine residues in apolipoprotein B-100 and cause its fragmentation [25]. Finally, the oxLDL exhibits strong negative charges, and will become LOX-1 ligand. Moreover, the binding and internalization of oxLDL in bLOX-1-CHO cells can be blocked by negatively charged compounds such as poly I or carrageenans, further supporting the importance of charge-dependent interactions between LOX-1 and its ligand [21].

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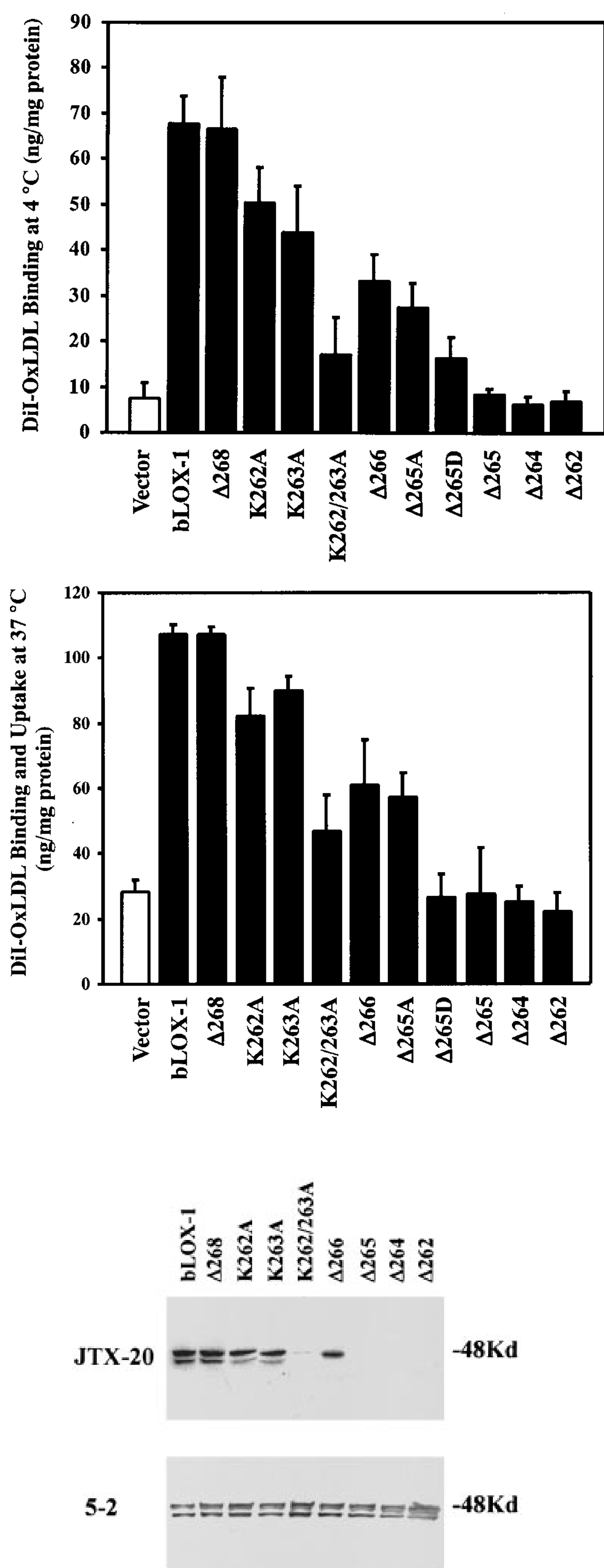

Figure 7 OxLDL binding and uptake in CHO cells transfected with wild-type and mutant bLOX-1 (as shown in Figure 6)

Top panel: oxLDL binding at 4 °C in native- and mutant-LOX-1-transfected CHO cells. Middle panel: oxLDL binding and uptake at 37 °C in native- and mutant-LOX-1-transfected CHO cells. The cells were incubated with 10 μg/ml DiI-labelled oxLDL for 3 h at 4 or 37 °C in the presence or absence of a 50-fold excess of unlabelled oxLDL. The specific binding was calculated by subtracting the values in the presence of an excess amount of unlabelled oxLDL. Data are means ± S.D. from four separate experiments. Bottom panel: Western-blot analysis of the LOX-1 mutants. Mutation of amino acid residues between 262 and 265 impairs recognition by the neutralizing antibody JTX-20, whereas recognition by antibody 5-2 was not changed. Immunostaining of the transfectants with JTX-20 exhibited the same results (results not shown).

Several cellular receptors that bind and internalize oxLDL have been identified and termed scavenger receptors (SRs), although the basic structures among them are different [18,19]. Many investigations have been performed to identify the specific determinants responsible for binding of oxLDL. They revealed that the lysine cluster in the collagen domain is the ligand-binding domain of the macrophage SR-AI/II [24,26,27]. The oxLDL-binding domain of CD36 was also localized in an immunodominant domain spanning amino acids 155–183 for several neutralizing antibodies [28]. Recently, Pearce et al. [29] reported that oxLDL binding to CD36 was sensitive to pH and ionic strength, indicating that electric interactions may be critical for binding. LOX-1 may bind oxLDL in a similar principle to these receptors through the positively charged residues including Lys-262 and Lys-263.

The second possible explanation is that the positive charge of Lys-262/Lys-263 is needed for the stabilization of tertiary structure of LOX-1. LOX-1 exhibits the highest protein sequence similarity to the members of group-II C-type animal lectins including natural killer (NK) cell receptors [20,30,31]. Although the topology for LOX-1 was not determined, recent studies have identified the crystal structure of lectin-like NK cell receptors [32,33]. The established structure of CD94, an NK cell receptor, may serve as a prototype for LOX-1. In the report on CD94, Boyington et al. [32] revealed that Lys-175 forms a salt bridge with Glu-104. The equivalent amino acids Lys-262/Lys-263 of LOX-1 may participate in the salt bridge with acidic amino acid pair such as Asp-182/Glu-183. Thus point mutations of the charged residues or deletion at the C-terminal end of the lectin domain could impair or disrupt the delicate structure of LOX-1, a prerequisite for ligand binding.

It is striking that the epitope for the LOX-1-neutralizing antibody precisely overlaps with the essential residues for oxLDL binding. This means that the essential C-terminal residues not only constitute a binding pocket for oxLDL, but are also exposed on the surface of the LOX-1 molecule and are accessible for antagonists. Since oxLDL is a large molecule of $\approx$ 300 kDa, the binding pocket of LOX-1 for oxLDL should be wide open. Although the precise tertiary structure of LOX-1 including stoichiometry with its ligand is unknown, targeting the C-terminal residues would be a good strategy to develop a LOX-1 antagonist.

In summary, we employed mutagenesis studies to analyse the structure–function relationship of LOX-1. The lectin-like domain of LOX-1 mediates oxLDL binding. Particularly, the conserved residues at the C-terminus of the lectin-like domain are essential for the binding activity. The results provide valuable information for designing a LOX-1 antagonist that is potentially important for establishing therapeutics for atherosclerosis.

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