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Report

Generation and characterization of chicken monoclonal antibodies against human LOX-1

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Abbreviations: LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; LDL, low-density lipoprotein; oxLDL, oxidized low-density lipoprotein; CTL, C-type lectin-like; DNP, 2,4-dinitrophenyl

Key words: LOX-1, oxLDL, chicken monoclonal antibody, chimeric antibody, neutralizing antibody

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Lectin-like oxidized low-density lipoprotein (LDL) receptor-1 (LOX-1) is the major receptor for oxidized LDL (oxLDL), and plays a key role in the pathogenesis of atherosclerosis and cardiovascular diseases. Monoclonal antibodies (mAbs) specific for human LOX-1 (hLOX-1) were generated by a phage display technique using chickens immunized with recombinant hLOX-1 (rhLOX-1). A total of 53 independent scFv clones reactive for rhLOX-1 were obtained. Of the 53 clones, 49 recognized the C-type lectin-like domain (CTL domain), which contributes to the binding of oxLDL. Of these, 45 clones inhibited oxLDL-binding with LOX-1. Furthermore, some of these clones cross-reacted with rabbit, pig and/or mouse LOX-1. For possible application as therapeutic agents in the future, two cross-reactive mAbs were re-constructed as chicken-human chimeric antibodies. The chimeric antibodies showed similar characteristics compared to the original antibodies, and inhibited oxLDL binding to LOX-1 expressed on CHO cells. The results obtained in this study indicate that anti-LOX-1 mAbs might be useful tools for functional analyses and development of therapeutic agents for cardiovascular indications such as atherosclerosis.

Introduction

LOX-1 was first identified in vascular endothelial cells, and has been characterized as the major receptor for oxLDL in endothelial cells.[1] Studies have indicated that LOX-1 has a critical role in the pathogenesis of atherosclerosis and cardiovascular diseases.[2] Recently, a soluble form of LOX-1 (sLOX-1) released by proteolytic cleavage was detected in serum from acute coronary syndrome (ACS) patients.[3] This suggests that sLOX-1 might be a useful biomarker for early diagnosis of ACS.

LOX-1 is a 50 kDa type-II membrane protein that, as assessed by structure, belongs to the C-type lectin family. LOX-1 consists of four domains, the N-terminal intracellular domain, the transmembrane domain, the Neck domain, and the CTL domain.[4] Among these, the CTL domain is critical for LOX-1 function, as the C-terminal residues and arginine residues in this domain are essential for oxLDL-binding.[5-7]

Although mAbs specific to LOX-1 are useful for expression and functional analyses of LOX-1,[1,5,8-11] the number of anti-LOX-1 mAbs is insufficient[1,5,9,11] at least in part because generation of mAbs against LOX-1 by immunization of mammalian species is difficult due to the high conservation of the CTL domain among mammals.[5]

However, the chicken is a useful animal for developing specific antibodies against conserved mammalian proteins because of the phylogenic differences between chickens and mammals.[12-15] In fact, numerous chicken mAbs have been produced using cell fusion and phage-display techniques.[12-15] Although a LOX-1 homolog has not yet been found in chickens, useful mAbs against mammalian LOX-1 can be produced by immunizing chickens. To study chicken mAbs against various LOX-1 epitopes, we generated 53 chicken mAbs specific to LOX-1 by a phage-display technique using chickens immunized with rhLOX-1. Here, we report data for 49 mAbs that recognized the CTL domain, of which 45 also inhibited oxLDL-binding with LOX-1.

Results

Production of recombinant LOX-1. Recombinant human, mouse, rabbit and pig versions of LOX (rhLOX-1, rmLOX-1, rrLOX-1 and rpLOX-1) as well as delta Neck, were each produced in FreeStyle^{TM} 293-F cells. These recombinant LOX-1 proteins were detected as approximately 62 kDa, 40 kDa, 20 kDa, 22 kDa and 30 kDa bands, respectively, on SDS-PAGE under non-reducing conditions (Fig. 1A). rhLOX-1 and delta Neck were each detectable

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as a half-molecule bands under reducing conditions (data not shown). These results confirmed that rhLOX-1 and delta Neck are cross-linked by a disulfide bond through Cys140.[7] The proteins exhibited binding activity toward human oxLDL, but not the negative control LDL (Fig. 1B). The result suggests that the recombinant proteins maintained the correct structure and function. rmLOX-1, rrLOX-1 and rpLOX-1 were monomers; these proteins showed the same profiles under both reducing and non-reducing conditions. Recombinant LOX-1s (human, mouse, rabbit and pig) were detected as broad bands or two bands (Fig. 1A). LOX-1s contains putative N-glycosylation signals,[5] so the differences in molecular weight (MW) between these bands are probably due to variation in glycosylation.

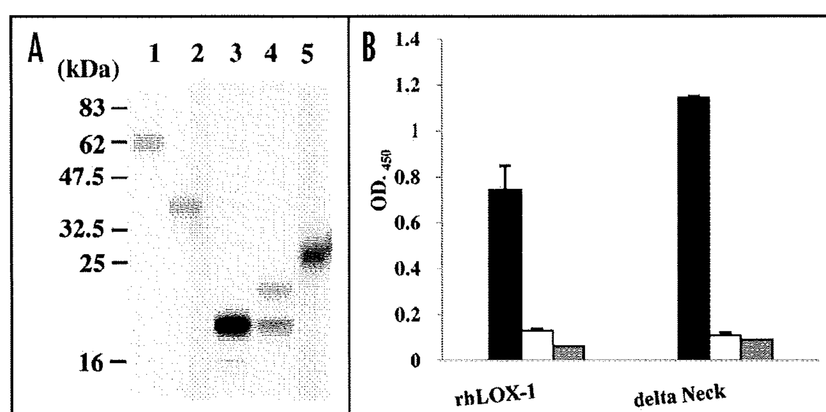

Figure 1. SDS-PAGE profiles and reactivity of recombinant LOX-1s. (A) SDS-PAGE profiles of rhLOX-1 (lane 1), delta Neck (lane 2), rmLOX-1 (lane 3), rrLOX-1 (lane 4) and rpLOX-1 (lane 5). Recombinant proteins were purified from the supernatant of 293-F cells by nickel affinity chromatography. All samples were subjected to SDS-PAGE under non-reducing conditions and were stained with CBBR. Numbers on the right indicate apparent molecular masses in kDa. (B) Reactivity of rhLOX-1 and delta Neck to oxLDL (black), LDL (negative control, white) and BSA (control, gray) was measured by ELISA using biotin-labeled rhLOX-1- or delta Neck-coated plates. Data are means ± SD of three independent experiments.

Specific antibodies against LOX-1. By using spleen cells from chickens immunized with rhLOX-1, the scFv phage library (5.0×10^8 cfu) was constructed. After the 6th round of panning selection against rhLOX-1, the specificity of the concentrated scFv phage library was examined by ELISA. Of 207 scFv phage clones from libraries of the 5th and 6th pannings, 113 were reactive for rhLOX-1 (data not shown). The results of nucleic acid sequencing in the positive clones showed that these clones could be subclassified to 51 independent clones (data not shown). In the panning selections against rmLOX-1 using the same phage-display library, 2 independent clones were selected from libraries of the 3rd and 4th pannings. Finally, a total of 53 clones were constructed as rIgY for quantitative experiments.

Reactivity of recombinant antibodies against LOX-1s. We investigated whether 53 chicken antibodies obtained by phage-display technique recognize the CTL or Neck domains and LOX-1 from other mammalian species.

The reactivities of the 53 rIgY antibodies against LOX-1 were assessed by ELISA and FACS. Of 53 clones, HUC5-34, HUC5-40, HUC6-34 and HUC6-41, reacted with rhLOX-1, but not with delta Neck (Fig. 2A). The result suggests that these four clones recognize the Neck domain of LOX-1 (anti-Neck domain clones). The residual clones reacted with both rhLOX-1 and delta Neck with similar intensities (data not shown), indicating that they recognize the CTL domain.

Seven (HUC52, HUC5-44, HUC5-53, HUC5-63, HUC5-90, HUC6-92 and HUC3-48) of 53 clones were cross-reactive with both rabbit and pig LOX-1 (Fig. 2B). HUC5-24 cross-reacted with rrLOX-1 (Fig. 2B), and HUC5-9 reacted with rpLOX-1 (Fig. 2B). The two clones (HUC3-1 and HUC3-48) selected from panning against rmLOX-1 were reactive with rmLOX-1. Interestingly, HUC3-48 reacted with LOX-1s from all species tested (Fig. 2B). However, in western blotting these clones did not react with recombinant LOX-1s (data not shown), indicating that these clones recognize conformational epitopes. In fact, these clones also reacted with LOX-1-expressing cells in FACS analysis (data not shown). None of the clones tested reacted with BSA and wild type CHO cells (data not shown).

Neutralization activity of anti-LOX-1 antibodies. LOX-1 is expressed in atherosclerosis and several cardiovascular diseases, such as myocardial ischemia.[2] In a rat model, administration of anti-LOX-1 antibody effectively suppressed intimal hyperplasia.[10] Thus, inhibition of LOX-1 activity may be a useful strategy to produce novel drugs for cardiovascular disorders. The neutralization activity of 53 anti-LOX-1 antibodies was examined using a modified method described previously.[19] Forty-five of 53 clones showed neutralization activity (data not shown), suggesting that these clones recognized the CTL domain, which is essential for oxLDL-binding.[5] Although the Neck domain of LOX-1 is not critical for oxLDL binding with LOX-1, anti-Neck domain clones HUC5-34, HUC5-40, HUC6-34 and HUC6-41 (Fig. 2A) slightly inhibited oxLDL-binding (data not shown). The MW of rhLOX-1 is about 62 kDa (Fig. 1A, lane 1), and that of rIgY is about 250 kDa.[17] With MW four-fold greater than rhLOX-1, anti-Neck domain clones likely exhibited steric inhibition.

To have potential as therapeutic agents, two clones, HUC52, which cross-reacted with rabbit and pig LOX-1, and HUC3-48, which recognized LOX-1s from all species tested, were re-constructed as chimeric IgG antibodies (Fig. 3). In order to avoid Ig effector functions involving complement activation and antibody-dependent cell-mediated cytotoxicity, the IgG4 subclass was selected. The chimeric antibodies had similar reaction patterns compared to their original rIgY forms (Fig. 4). Inhibition studies were then performed using LOX-1-expressing CHO cells. Regardless of the animal species tested, the two chimeric IgGs reacted with LOX-1-expressing CHO cells (Fig. 5A), and blocked oxLDL-binding to LOX-1-expressing CHO cells (Fig. 5B). HUC3-48 also showed inhibition activity against mouse LOX-1-expressing cells (data not shown).

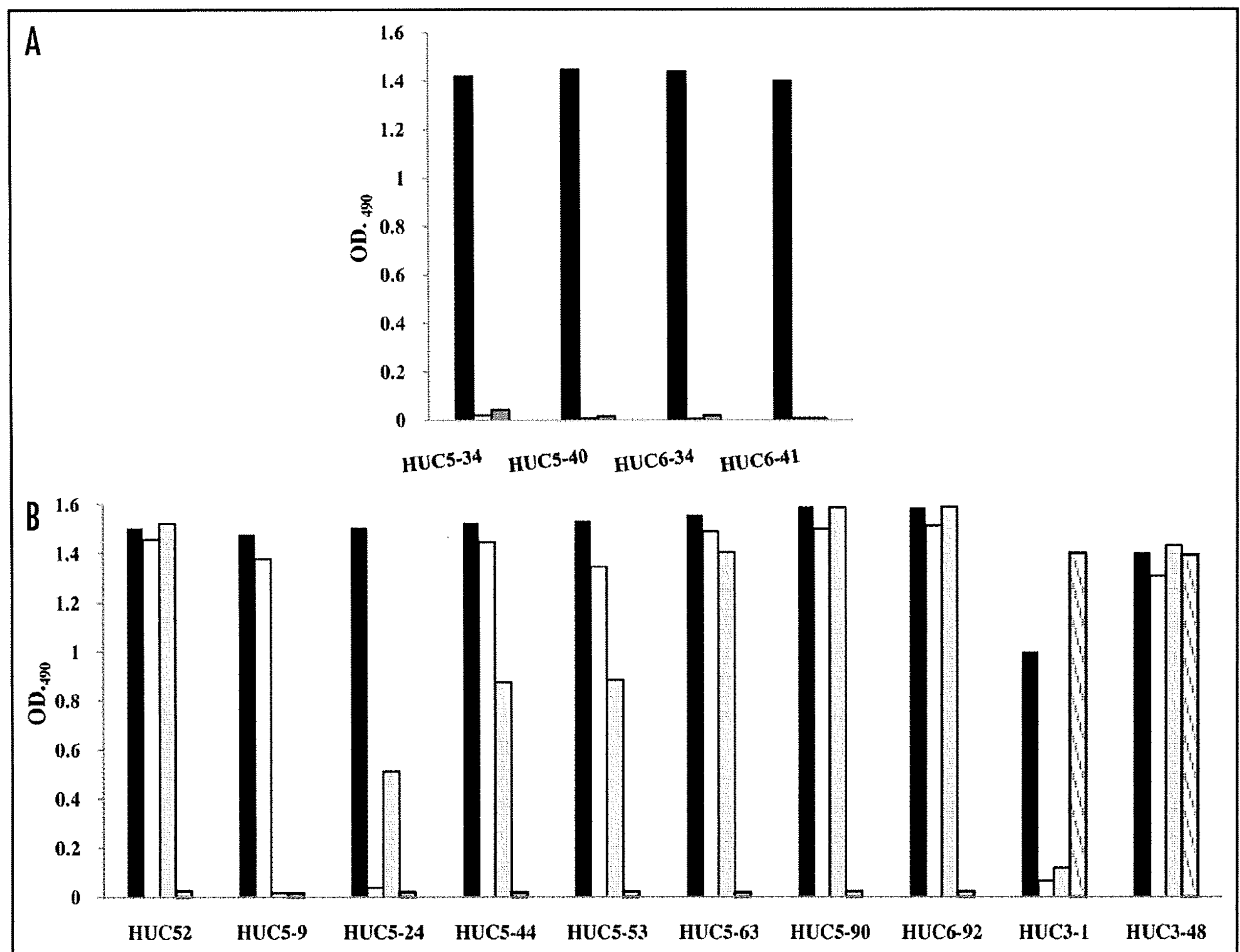

Figure 2. Reactivity of rIgY antibodies against recombinant LOX-1s. (A) Reactivity of rIgY antibodies against rhLOX-1 and delta NECK. rIgYs (1 μg/ml) were added to wells coated with biotinylated rhLOX-1 (black), delta Neck (white) or BSA (negative control, gray). (B) Cross-reactivity of 53 rIgY antibodies against LOX-1 from four species. The rIgYs (1 μg/ml) were added wells coated with biotinylated rhLOX-1 (black), rpLOX-1 (white), rrLOX-1 (gray) or rmLOX-1 (shaded). Of the 53 clones, eight cross-reacted with rrLOX-1 and/or rpLOX-1 and only two clones also recognized rmLOX-1.

Discussion

LOX-1 is the main receptor for oxLDL on endothelial cells, and it mediates the recognition and internalization of oxLDL.[1] Recent studies on LOX-1 have shown that this molecule plays a critical role in the development of atherosclerosis and cardiovascular diseases.[2] For further research into the function of LOX-1, LOX-1-specific mAbs are thought to be one of the most useful tools for basic analysis and clinical applications. However, a limited number of mAbs against human LOX-1 have been reported. This might be because the CTL domain is highly conserved among mammalian species[5] and so anti-LOX1 mAbs are difficult to generate. We have successfully produced chicken mAbs against conserved mammalian molecules using cell fusion and phage-display techniques.[12-15] In the present study, a total of 53 scFv chicken mAbs specific for LOX-1 were generated from only two panning selections. Most of the rIgY forms from these scFv antibodies recognized the CTL domain of LOX-1, and only 4 clones recognized the Neck domain (Fig. 2A). These results indicate that the chicken is a useful animal for producing antibodies specific for mammalian LOX-1, particularly the CTL domain.

Mice, rabbits and pig are typically used as models for atherosclerosis and cardiovascular diseases. For example, ApoE-knockout mice and Watanabe heritable hyperlipidemic rabbits are used as animal models of spontaneous hyperlipidemia and in the analysis of LOX-1 function.[16,18-21] Therefore, we investigated whether the mAbs presented here cross-react with LOX-1s from these model animals. Six clones (HUC52, HUC5-44, HUC5-53, HUC5-63, HUC5-90 and HUC6-92) that reacted with rhLOX-1 also displayed cross-reactivity to both rrLOX-1 and rpLOX-1 (Fig. 2B). In contrast, no rmLOX-1 cross-reactive antibodies were obtained in the first antibody selection. We then selected antibodies using the scFv phage library from rhLOX-1-immunized chickens, and identified clones HUC3-1, which cross-reacted with rmLOX-1 and rhLOX-1, and HUC3-48, which cross-reacted with recombinant LOX-1s from all three species examined (Fig. 2B).

A total of 45 rIgYs including HUC3-48 mAb inhibited oxLDL-binding with LOX-1 (data not shown). The result indicates that these 45 mAbs are reactive for the CTL domain, which is critical for the binding of oxLDL.[5]

For possible utilization as therapeutic antibody agents for humans, we reconstructed two clones as chicken-human chimeric IgG by converting the chicken constant regions into human regions (Fig. 3). These antibodies were evaluated in inhibition assays using cells expressing LOX-1. HUC52 and HUC3-48 chimeric IgGs, which had similar LOX-1 reactivity compared to their respective parental antibodies (Fig. 4), blocked oxLDL binding to LOX-1 expressed on CHO cells (Fig. 5B). This evidence suggests that these antibodies should be further evaluated in animal models.

Since the variability of FR residues in chicken antibodies is very small compared to those in human and rodent, it is possible that the same human template can be used to humanize all chicken antibodies. Humanization of chicken mAbs has been achieved by CDR-grafting, followed by framework fine-tuning using a phage displayed combinatorial library.[22] In the current study, chicken mAbs were successfully humanized as divalent IgG4 without loss of antibody affinity. Therefore, humanized chicken antibodies may have applications as treatments for human disease in the future.

In conclusion, we generated 53 mAbs against LOX-1 using phage-display techniques and 45 of these mAbs that recognized the CTL domain were neutralizing antibodies. In addition, two human chimeric mAbs from HUC52 and HUC3-48, cross-reacted with rabbit, pig or mouse LOX-1, also showed neutralization activitities against LOX-1 expressing cells. These results indicate that our mAb clones might be useful tools for the investigation of LOX-1 function, and might have clinical applications. Production of chicken-mouse, chicken-rabbit and chicken-pig chimeric mAbs for preclinical studies using animal models is in progress.

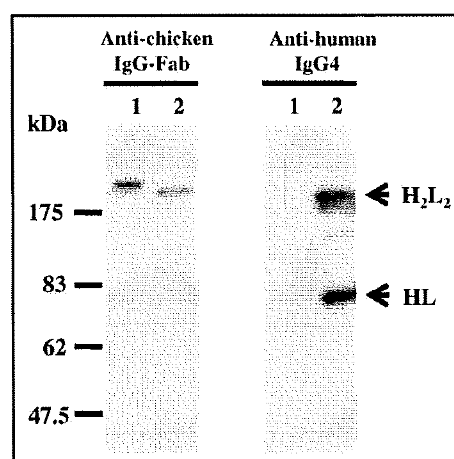

Figure 3. Western blot analysis of recombinant chicken antibody and chicken-human chimeric antibody. The rIgY (lane 1) and chimeric IgG (lane 2) were each run on 10% SDS-polyacrylamide gels under non-reducing conditions. Anti-chicken IgG-Fab and anti-human IgG4 were used as a detection antibody. Numbers on the right indicate apparent molecular masses in kDa. H_2L_2 represents antibody full length and HL indicates the halved molecule.

Materials and Methods

Antigens. Recombinant human LOX-1 (amino acids 61–273; rhLOX-1) and its deletion mutant (amino acids 137–273; delta Neck), recombinant mouse LOX-1 (amino acids 188–363 of mouse LOX-1; rmLOX-1), recombinant rabbit LOX-1 (amino acids 101–278 of rabbit LOX-1; rrLOX-1) and recombinant pig LOX-1 (amino acids 61–274 of pig LOX-1; rpLOX-1) were generated with pcDNA4/myc-HisA (Invitrogen, USA) in order to synthesize each LOX-1 as a 6xHistidine tag (His tag) fusion protein as described previously.[18] Proteins were produced in a FreeStyle™ 293 Expression System (Invitrogen) and were purified using a Probond protein purification kit (Invitrogen), and their molecular sizes were confirmed by SDS-PAGE. Purified proteins were also biotinylated using a Biotin Labeling Kit-NH_2 (Dojindo, Japan). The PCR primers used in this study were shown in Table 1.

Cells. cDNA encoding the human LOX-1 was subcloned into a Tet-On Gene Expression vector pTRE2 hyg (Clontech Laboratories, USA). The plasmid was transfected into CHO-K1 Tet-On cells

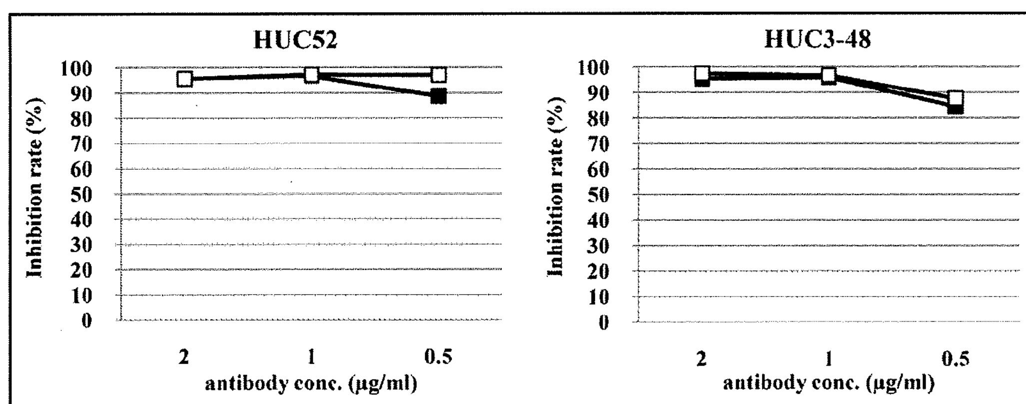

Figure 4. Inhibition of oxLDL-binding with LOX-1 by anti-LOX-1 antibodies. rhLOX-1 was used as the capture molecule. rIgY (closed square) or chimeric IgG (open square) indicated inhibition rate of oxLDL-binding with LOX-1 using anti-human ApoB antibody. Anti-DNP rIgY was used as a negative control and standard for comparison.

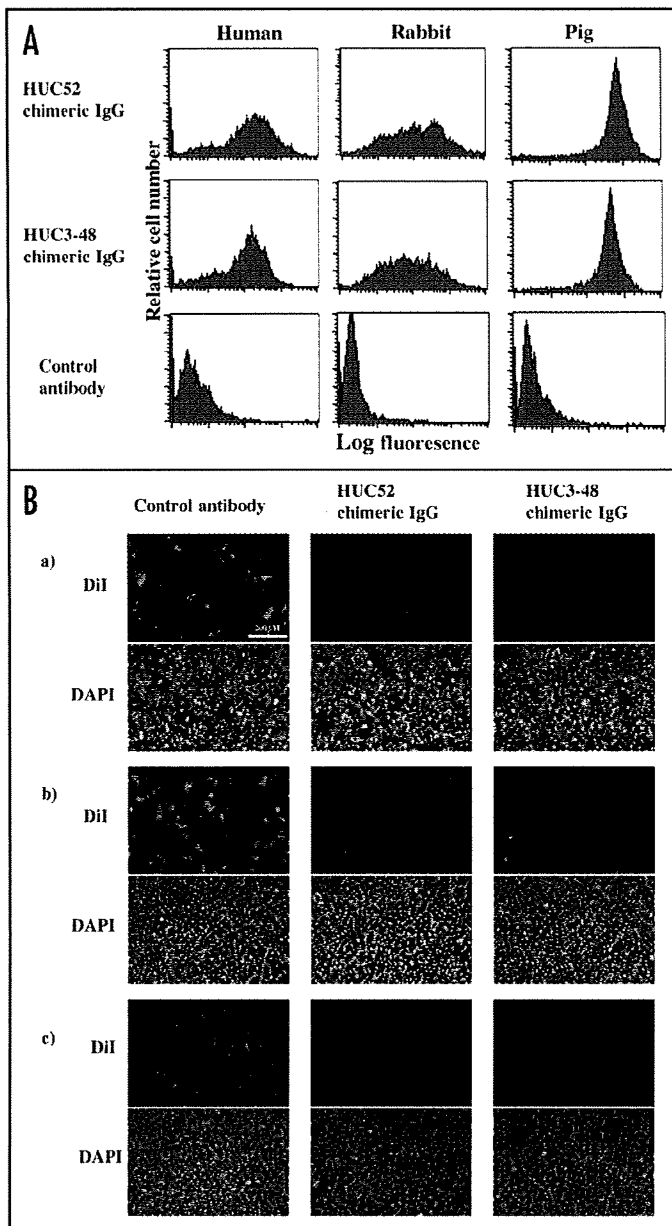

Figure 5. Reactivities and blocking activities of chimeric IgGs against LOX-1-expressing cells. (A) LOX-1-expressing CHO cells were incubated with chimeric IgG (1 μg/ml) revealed with FITC-labeled anti-human IgG. Fluorescence was analyzed by FACS. (B) Human (a), rabbit (b) or pig (c) LOX-1-expressing CHO cells were incubated with chimeric IgG (5 μg/ml). DiI-labeled oxLDL (3 μg/ml) was added for 2 h. After that, the cells were fixed with 4% (v/v) paraformaldehyde. Then, the nuclei of the cells was counterstained with DAPI and subjected to observation with fluorescence microscopy.

(Clontech Laboratories) by Lipofectamin2000 tansfection reagent (Invitrogen) according to the manufacture's instruction. Stably transformants were selected under Ham's F12/10% fetal bovine serum (FBS) supplemented with 400 μg/ml hygromycin B (Wako, Japan). The cells expressing LOX-1 in response to doxycycline (Wako) were selected. The LOX-1 expression was induced with 1 μg/ml doxycyline for 24 h before the experiments. Rabbit and pig cDNA were subcloned into a mammalian expression vector *p*EF6V5-HisA (Invitrogen), respectively. The plasmid was each transfected into CHO-K1 cells by FuGENE HD transfect reagent (Roche Diagnostics, Switzerland). Stably LOX-1-expressing cells were selected and maintained under Ham's F12/10% FBS supplemented with 8 μg/ml blasticidin S (Invitrogen).

Immunization and construction of phage-display library. Chicken scFv mAbs were generated by the chicken phage-display technique.[15] One-month-old H-B15 inbred chickens were immunized intraperitoneally (i.p.) with rhLOX-1 (50 μg/ml/chicken) in an equal volume of alum solution (ALUM). The chickens received three additional i.p. injections with the same antigen together with ALUM at 3-week intervals. Four days after the final injection, spleen cells were isolated from immunized chickens. RNA was extracted from spleen cells, immunoglobulin variable region (VH and VL) genes were amplified and a scFv phage library was constructed as described previously.[15]

Panning selection. The phage-display scFv library from rhLOX-1-immunized chickens was panned against rhLOX-1 or rmLOX-1. For selection of rhLOX-1-specific antibodies, 100 μl (10 μg/ml) of rhLOX-1 was coated on a Maxisorp Nunc-Immuno™ module (NUNC, USA). An ELISA plate was then blocked with 2% (w/v) non-fat dried milk powder (EuroClon, Italia) in phosphate-buffered saline (PBS) at room temperature (RT) for 1 h. For selection against rmLOX-1, 100 μl (5 μg/ml) of biotin-labeled rmLOX-1 was coated on Nunc Immobilizer™ Streptavidin plates (NUNC). Panning selection was performed as described previously.[15]

Recombinant chicken IgY (rIgY) and chicken-human chimeric IgG4 antibody (chimeric IgG). The rIgY and chimeric IgG were generated using the VH and VL genes from phage-displayed chicken antibodies obtained in this study, and plasmid vectors[17,23] were used for construction of the light and heavy chains, respectively. Constructed plasmid DNAs were transfected into COS-7 cells or FreeStyle™ 293-F cells (Invitrogen) using FuGENE HD transfect reagent.

Western blotting. Western blotting for detection of recombinant LOX-1s was carried out. Recombinant LOX-1s were subjected to SDS-PAGE under non-reducing conditions, and were then transferred to an Immun-Blot™ PVDF membrane (Bio-Rad, USA) at 350 mA for 1 h. Membranes were incubated for 1 h at room temperature with chicken anti-LOX-1 rIgYs and developed by ECL plus (GE Healthcare, UK). Chemiluminescent signals were then analyzed using a LAS-3000 (Fuji Film, Japan).

Western blotting for detection of rIgY and chimeric IgG was carried out as described previously.[17,18] Horseradish peroxidase (HRP)-labeled anti-chicken IgG-Fab fragment (Bethyl, USA) was used for detection of rIgY and chimeric IgG. Mouse anti-human IgG4 antibody (BD Biosciences, USA) was used as the first antibody and HRP-labeled-mouse IgG antibody (Southern Biotech, USA) was used as the second antibody for detection of chimeric IgG. The rIgY and chimeric IgG were detected using ECL plus and LAS-3000.

ELISA for reactivity and cross-reactivity of rIgYs. The wells of Nunc Immobilizer™ Streptavidin plates were coated with 50

μl (1 μg/ml) of biotin-labeled rhLOX-1, delta Neck, rmLOX-1, rrLOX-1, rpLOX-1 or BSA (control antigen) in carbonate buffer (pH 9.5) for 1 h at RT. After washing with PBS-T, the respective rIgYs were added at 1 μg/ml. Plates were incubated at 37°C for 1 h. After washing with PBS-T, bound antibodies were detected using a HRP-labeled goat anti-chicken IgG (H + L) (Kirkeggaard and Perry Laboratories, USA). After washing with PBS-T, *o*-phenylene diamine sulfate (OPD, Sigma, USA) was added and the optical density was measured at 490 nm using a Model 680 microplate reader (Bio-Rad). Human anti-human LOX-1 mAb TS-92,[9] was used as a positive control.

FACS analysis. The recombinant antibodies were each incubated with LOX-1-expressing CHO cells at 4°C for 1 h in PBS containing 0.1% FBS and 0.1% NaN_3 (FACS buffer). After washing with FACS buffer, cells were incubated with FITC-labeled anti-chicken IgG (H + L) or anti-human IgG (H + L) (Southern Biotech, USA) for 30 min at 4°C. Fluorescence was analyzed by FACSCalibur (BD, USA).

Inhibition analysis by mAb in oxLDL-binding with recombinant LOX-1protein. Inhibition analysis was performed using a modified method reported previously.[16] Biotin-labeled rhLOX-1 (50 ng/well) was immobilized on Nunc Immobilizer™ Streptavidin plates by incubating at RT for 1 h in 50 μl of carbonate buffer. After washing with PBS, rIgY (1 μg/ml), TS-92 (positive control, 1 μg/ml) and anti-2,4-dinitrophenyl (DNP) rIgY (negative control, 1 μg/ml)[18] were each added to rhLOX-1-coated wells, and plates were incubated at RT for 2 h. After washing with PBS, plates were incubated overnight at 4°C with 50 μl of human oxLDL (3 μg/ml) in PBS containing 20% (vol/vol) newborn calf serum (NBCS, Gibco, USA). Plates were then washed with PBS, and incubated for 2 h at RT with the HRP-sheep anti-human ApoB polyclonal antibody (The Binding Site, UK) diluted 1,000 times with PBS containing 1% (w/v) BSA for detection of oxLDL. After washing with PBS, the peroxidase reaction was initiated by incubating plates for 5 min at RT with 50 μl of SureBlue Reserve™ TMB Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories, USA). The reaction was terminated with 0.1 M hydrochloric acid and 0.3 M sulfuric acid. Peroxidase activity was determined by measuring absorbance at 450 nm.

Inhibition analysis by mAb in oxLDL-binding with LOX-1expression cells. The LOX-1-expressing CHO cells were incubated with anti-LOX-1 antibody in Ham's F12 containing 10% NBCS for 1 h at 37°C. After 1 h, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes, USA)-labeled oxLDL (3 μg/ml) was added to the cells for 2 h. The cells were washed three times with PBS and fixed with 4% (v/v) paraformaldehyde in PBS for 20 min. Then, the nuclei of the cells were counterstained with 5 μg/ml DAPI (Sigma) and subjected to observation with fluorescence microscopy.

Table 1 PCR primers used in this study

Primer	Sequence
Leader-F	5'-ATGCGGATCCGCCATGGCCTGGGCTCCTCTCCT
Leader-R	5'-TGCCTGCACCAGGGAACCTG
hLOX-F	5'-TCCCTGGTGCAGGCATCCCAGGTGTCTGACCTC
hLOX-R	5'-ATGCACCGGTCTGTGCTCTTAGGTTTGCC
hLOX-Neck-F	5'-TCCCTGGTGCAGGCAGTAGCAAATTGTTCAGCTC
mLOX-F	5'-TCCCTGGTGCAGGCAGAGTCCCAGAGAGAACTC
mLOX-R	5'-ATGCACCGGTAATTTGCAAATGATTTGTC
rLOX-F	5'-TCCCTGGTGCAGGCAGAGTCACAAAGGGAACTC
rLOX-R	5'-ATGCACCGGTCTCTGATCTCAGCAGATTTG
pLOX-F	5'-TCCCTGGTGCAGGCATCCCAGGTGTCTGATCTCCTG
pLOX-R	5'-GACTACCGGTCTGTGCTCTCAAGAGATTCGC

Primers for generation of recombinant LOX-1 protein. Restriction sites are underlined.

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Suppression of Choroidal Neovascularization in Lectin-like Oxidized Low-Density Lipoprotein Receptor Type 1–Deficient Mice

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Purpose. To elucidate the role of the scavenger receptor, lectin-like oxidized low-density lipoprotein receptor type 1 (LOX-1), in the formation of choroidal neovascularization (CNV).

Methods. CNV was induced by laser photocoagulation of the ocular fundus in mice. The expression of LOX-1 mRNA and protein after laser injury was determined by real-time RT-PCR and Western blot analysis. Gelatin zymography was used to measure the activity of matrix metalloproteinase (MMP)-2 and pro-MMP-9, and ELISA was used to determine monocyte chemoattractant protein (MCP)-1 and vascular endothelial growth factor (VEGF) levels. At 14 days after laser injury, the extent of CNV was evaluated by fluorescein angiography and lectin staining using confocal microscopy.

Results. In wild-type mice, the relative expression level of LOX-1 mRNA compared with the control increased significantly 6 hours after laser injury and peaked 12 hours after laser injury ($P = 0.011$ and $P = 0.0006$, respectively), and the expression of LOX-1 protein was also detected 1 and 3 days after laser injury. Increases in MMP-2, pro-MMP2, and pro-MMP-9 after laser injury were reduced in LOX-1-deficient mice compared with wild-type mice. At 3 days after laser injury, increases in MCP-1 and VEGF significantly decreased in LOX-1-deficient mice compared with wild-type mice ($P = 0.014$ and $P = 0.001$, respectively). Morphometric analyses revealed that the induction of CNV formation was significantly inhibited in LOX-1-deficient mice.

Conclusions. These results suggest that LOX-1 plays an important role in the formation of CNV. This scavenging system might thus be a novel therapeutic target for CNV. (*Invest Ophthalmol Vis Sci.* 2009;50:3970–3976) DOI:10.1167/iovs.07-1177

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Age-related macular degeneration (AMD) is the leading cause of legal blindness in persons older than 55 in developed countries.[1] Late AMD is subdivided into two forms, atrophic and (neovascular) exudative. The latter is associated with the most severe cases of visual loss caused by the growth of abnormal new vessels under the retinal pigment epithelium (RPE) from the choroid, leading to choroidal neovascularization (CNV). The pathogenesis of AMD is regarded as multifactorial, with age, genetic background, environmental risks, and systemic conditions playing important roles in its progression.[2–4] Although adhesion molecules, cytokines, and growth factors have been identified as contributing factors, the molecular mechanisms relating to AMD pathogenesis are not well understood.

Recently, similarities have been suggested between the pathogenesis of AMD and atherosclerosis.[5,6] During the progression of atherosclerosis, oxidized low-density lipoprotein (ox-LDL) and its specific receptors, so-called scavenger receptors (SRs), play a critical role in foam-cell formation after endothelial dysfunction and macrophage recruitment.[7] Interestingly, a previous clinical study identified elevated plasma levels of ox-LDL in patients with exudative AMD,[8] whereas the expression of ox-LDL SRs has been observed in surgically excised CNV.[8,9] Furthermore, several studies have shown that matrix metalloproteinases (MMPs) and chemokines, such as monocyte chemoattractant protein (MCP)-1, are involved in the remodeling and recruitment of leukocytes in atherosclerosis and CNV.[10–13]

Lectin-like ox-LDL receptor type 1 (LOX-1) is a recently identified SR expressed by vascular endothelial cells[14] that plays an important role in the formation of in vivo atherogenesis.[15] The induced expression of LOX-1 and its association with oxidative stress might also contribute to the formation of CNV in patients with AMD. Indeed, we previously demonstrated LOX-1 expression in surgically obtained CNV specimens from patients with AMD and other diseases.[16] Animal experiments have also revealed that LOX-1 is involved in inflammatory reactions of the eye through its regulation of leukocyte-endothelial interactions. Recent research into retinal angiogenic disorders has suggested that the inflammatory reaction is important in deterioration of the neovascular lesions, whereas the upregulated expression of LOX-1 in vascular endothelial cells has been shown to induce MMPs and MCP-1 expression.[17,18]

We hypothesized that elucidation of the potential roles and associated molecular mechanisms of LOX-1 could lead to the development of novel therapeutic modalities for AMD and other retinal angiogenic disorders. Here we report the upregulation of LOX-1 mRNA and protein expression in a mouse CNV model and suggest that LOX-1 and associated factors (such as

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MMP-2, MMP-9, and MCP-1) contribute to the formation of CNV.

Methods

Laser-Induced CNV in Mice

The generation and genotyping of LOX-1-deficient mice (on a C57BL/6 background) has been previously described.[15] The present study used 8-week-old C57BL/6 and LOX-1-deficient male mice. Laser-induced CNV was performed as described previously, with minor modifications.[19] Briefly, mice were anesthetized by intraperitoneal injection of 0.3 mL ketamine hydrochloride diluted (1:10) with sterile water. The pupils of the animals were dilated with 1% tropicamide, and krypton laser photocoagulation (spot size, 50 μm; duration, 0.05 second; power, 400 mW) burns were made to each retina using a slit lamp delivery system and a coverglass as a contact lens. For analysis of incident and extension of laser-induced CNV, CNV was induced in mice by three or four burns at the 6, 9, 12, and 3 o'clock positions around the optic disc. Any mouse with a hemorrhage or without an evident bubble (the sign of ruptured Bruch's membrane) was excluded from further analysis. The animals were maintained in a 12-hour light/12-hour dark cycle and had free access to food and water. All the mice were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Real-Time Reverse Transcription–Polymerase Chain Reaction

At 6 hours, 12 hours, 1 day, 2 days, 7 days, and 14 days after laser injury (10 burns), the eyes were dissected immediately, and total RNA was isolated from the posterior segment with an RNA isolation kit (AquaPure; Bio-Rad Laboratories, Hercules, CA). To remove genomic DNA, the total RNA preparation was treated with DNase-I (Invitrogen, Carlsbad, CA). Assay-on-demand primers and probes systems (Applied Biosystems, Foster City, CA) were used to quantify the mRNAs for a mouse LOX-1 assay (ID Mm00454586) and an 18S ribosomal RNA (rRNA) assay (ID Hs99999901). Real-time RT-PCR was performed with 10 ng total RNA on a sequence detection system (ABI Prism 7000; Applied Biosystems) with an RT-PCR system (SuperScript One-Step; Gibco BRL, Grand Island, NY). The threshold cycle of fluorescence units was evaluated to quantify mRNA levels, which were normalized according to the 18S rRNA levels and were expressed as the mean ± SD, as previously described.[20] Eyes with no laser injury were used as a control. The experiment was repeated three times.

Gelatin Zymography

At 3, 5, 9, and 21 days after laser injury (10 burns), the eyes were enucleated and analyzed by gelatin zymography using a commercially available electrophoresis kit (Gelatinzymo; Yagai Research Center, Yamagata, Japan), as previously described.[21] Briefly, equal amounts of protein (10 μg) were mixed with buffer (50 mM Tris-HCl buffer [pH 6.8] containing sodium dodecyl sulfate [SDS], glycerol, and bromophenol blue) and were electrophoresed. Supplied markers containing active MMP-2, pro-MMP-2, and pro-MMP-9 were also loaded onto the gel as references. After electrophoresis, the gels were agitated for 30 minutes in Triton X-100 buffer and shaken for 30 minutes in 50 mM Tris-HCl buffer (pH 7.5) containing NaCl to restore enzymatic activity. Samples were then incubated in 50 mM Tris-HCl buffer (pH 7.5) containing 200 mM NaCl and 5 mM $CaCl_2$ at 37°C for 26 hours to allow proteolysis of the gelatin. Subsequently, the gels were stained for 30 minutes with Coomassie blue and were destained in 30% methanol and 5% acetic acid. The experiment was repeated three times. Determination of the band intensity was analyzed by ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html), as previously described.[21]

Western Blot Analysis

At 1, 3, and 7 days after laser injury (10 burns), the eyes were enucleated, and the posterior segment of each was homogenized in radioimmunoprecipitation assay (RIPA) buffer. The homogenates were centrifuged at 20,000*g* for 15 minutes at 4°C, and the protein concentrations in the supernatants were determined using a DC protein assay kit (Bio-Rad Laboratories). Equal amounts of protein (10 μg/lane) were separated on 10% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 and 5% bovine serum albumin and were incubated overnight at 4°C with 1:200 dilutions of primary antibody against mouse LOX-1, as previously described,[15] and then with a peroxidase-linked second antibody (Abcam, Cambridge, UK). Chemiluminescence was detected with an enhanced chemiluminescence Western blot analysis kit (Amersham Pharmacia Biotech). The experiment was repeated three times.

Enzyme-Linked Immunosorbent Assay for MCP-1 and VEGF

At 3 days after laser injury (15 burns), the eyes were enucleated and the RPE-choroid-sclera complex was isolated and homogenized in 250 μL RIPA buffer. Homogenates were centrifuged at 20,000*g* for 15 minutes at 4°C, and protein concentrations in the supernatants were determined as mentioned. MCP-1 and VEGF levels in the lysate were determined by a mouse MCP-1 or VEGF ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol and were normalized to total protein as previously described.[22,23]

Incidence and Extension of Laser-Induced CNV

Incidences of CNV were determined by fluorescein angiography as previously described, with minor modifications.[19,24] At 14 days after laser injury (four burns), the lesions were studied by fluorescein angiography to evaluate CNV development and activity. Briefly, after intraperitoneal injection of 0.3 mL of 1% fluorescein sodium (Alcon, Tokyo, Japan), fluorescein angiography was performed at early and late phases using a scanning laser ophthalmoscope (SLO101; Rodenstock, Munich, Germany).

In addition, the mice were perfused with 20 mL phosphate-buffered saline (PBS) containing 50 μg/mL fluorescein-labeled tomato lectin (Vector Laboratories, Burlingame, CA) to stain the blood vessels. The eyes were harvested and fixed in 4% paraformaldehyde, and flatmounts of the RPE-choroid-sclera were prepared and stained for elastin using elastin ab (Sigma) followed by a Cy3-labeled secondary antibody (Sigma) as previously described.[25] The extent of laser-induced CNV was determined by confocal laser scanning microscopy as the area of fluorescein labeling in the flatmounts. Histologic images were captured, and pixels were measured using graphics software (Adobe Photoshop, version 7.0; Adobe Systems Inc., San Jose, CA) and ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html), according to the tutorial.

Statistical Analysis

Statistical comparisons of multiple groups were performed by one-way analysis of variance (ANOVA) followed by Fisher pairwise least significant difference (PLSD) test. Comparisons of two groups were made using either the χ^2 test or the Student *t*-test. $P < 0.05$ was considered statistically significant.

Results

LOX-1 mRNA and Protein Expression in Laser-Induced CNV

Real-time RT-PCR analysis using total RNA derived from mouse retina-RPE-choroid-sclera tissue was performed to quantify the

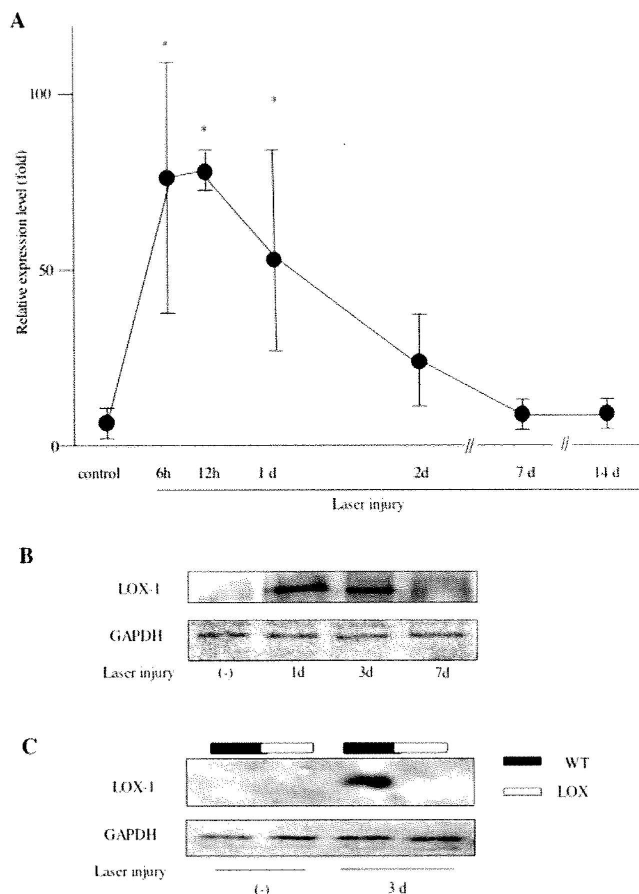


FIGURE 1. Real-time RT-PCR analysis of LOX-1 mRNA expression in posterior segment extract samples after laser injury (**A**). LOX-1 mRNA was standardized with 18S rRNA and expressed as the mean ± SD (n = 3). Data were analyzed using ANOVA. *Asterisks*: statistically significant differences ($P < 0.05$) compared with the control. Western blot analysis for LOX-1 in posterior segment extracts (**B**, **C**). LOX-1 expression was detected 1 day and 3 days after laser injury in wild-type mice (**B**). LOX-1 expression was not detected without laser injury in wild-type mice and LOX-1-deficient mice. The expression of LOX-1 was also not detected in LOX-1-deficient mice 3 days after laser injury (**C**). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein levels were included as a control.

relative levels of LOX-1 mRNA expression after laser injury. The expression level showed a transient peak between 6 and 12 hours after injury and then gradually returned to basal levels during the next 2 weeks (Fig. 1A). Semiquantitative analysis revealed that the relative expression of LOX-1 mRNA significantly increased 6 hours after laser injury by 71.5 ± 48.8-fold and peaked 12 hours after injury at 77.0 ± 7.7-fold compared with the control ($P = 0.011$ and $P = 0.0006$, respectively). At 1 day after injury, relative LOX-1 expression was significantly different from that of the control (52.8 ± 35.0-fold; $P = 0.01$). At 2, 7, and 14 days after injury, the relative LOX-1 expression was not significantly different from that of the control (32.2 ± 15.3-fold, $P = 0.09$; 6.7 ± 4.3-fold, $P = 0.75$; 6.8 ± 3.4-fold, $P = 0.74$, respectively). To confirm the expression of LOX-1 protein, Western blot analysis was performed. LOX-1 protein expression was clearly detected 1 day and 3 days after laser injury in wild-type mice (Fig. 1B) but was not detected in wild-type and LOX-1-deficient mice without laser injury. It also was not detected in LOX-1-deficient mice 3 days after laser injury (Fig. 1C).

Laser-Induced CNV in LOX-1–Deficient Mice

Our preliminary survey suggested that LOX-1-deficient mice undergo normal development of the ocular tissues and many organs, as observed previously.[15] Morphologic studies of LOX-1-deficient mouse eyes showed normal vasculature in the retinal and choroidal tissues and no abnormalities in the cornea, lens, iris, ciliary body (data not shown), and ocular fundus (Figs. 2A, B). To further determine the role of LOX-1, we compared the formation of laser-induced CNV between LOX-1-deficient and wild-type mice. At 14 days after laser injury, hematoxylin and eosin staining revealed proliferative membranes in the middle area of the lesion underlying the RPE and choroid in wild-type mice (Fig. 2C). By contrast, in LOX-1-deficient mice, fusiform membranes were rare and were primarily observed underlying the choroid (Fig. 2D).

Incidences of laser-induced CNV were determined by fluorescein angiography, which was performed 14 days after laser injury (Fig. 3). In wild-type mice, dye leakages, which were identified by the presence of hyper-fluorescein spots that became larger over time, were observed in 27 (93.0%) of the 29 burns. By contrast, dye leakage was significantly less common in LOX-1-deficient mice and was observed in 14 (58.3%) of the 24 burns ($P = 0.003$).

Perfusion of fluorescein-labeled tomato lectin was used to stain the vascular endothelium to measure the size of the laser-induced CNV, which was calculated from digitally captured images of RPE-choroid-sclera flat-mounts (Fig. 4A). The extent of CNV was significantly reduced in LOX-1-deficient mice compared with wild-type mice ($P = 0.011$; Fig. 4B), further confirming that laser-induced CNV was inhibited in LOX-1-deficient mice.

Induction and Activation of MMP-2 and MMP-9 Proteins

We next investigated the induction of MMP-2, pro-MMP-2, and pro-MMP-9 with gelatin zymography. In wild-type mice, the induction of pro-MMP-2 and pro-MMP-9 was clearly evident 3, 5, 9, and 21 days after laser injury (Fig. 5); the activated form of MMP-2 was also more conspicuous than basal level.

Similar experiments with LOX-1-deficient mice revealed only faint bands for MMP-2, pro-MMP-2, and pro-MMP-9. Densitometric analysis of the bands revealed that the induction of pro-MMP-9 in wild-type mice significantly increased 3 and 5 days after laser injury by 4.6 ± 1.3- and 4.6 ± 1.9-fold compared with the control ($P < 0.001$ and $P < 0.015$, respectively). The induction of pro-MMP-9 in LOX-1-deficient mice also increased 3 days after laser injury by 1.7 ± 0.5-fold compared with the control ($P = 0.022$).

There was a significant difference between wild-type mice and LOX-1-deficient mice with the induction of pro-MMP-9 at 3 and 5 days after laser injury ($P = 0.02$ and $P = 0.032$, respectively). In wild-type mice, the induction of pro-MMP-2 was significantly increased 3, 5, 9, and 21 days after laser injury by 14.7 ± 3.3-, 21.2 ± 5.2-, 16.6 ± 5.9-, and 13.4 ± 4.7-fold compared with the control, and the induction of MMP-2 was also increased 3, 5, 9, and 21 days after laser injury by 4.0 ± 1.0-, 5.4 ± 1.2-, 4.4 ± 0.7-, and 2.2 ± 1.0-fold compared with the control. By contrast, in LOX-1-deficient mice, the induction of pro-MMP-2 was increased 3, 5, and 9 days after laser injury by 8.4 ± 1.4, 6.7 ± 2.5-, and 5.9 ± 3.1-fold compared with the control, and the induction of MMP-2 also increased 3, 5, and 9 days after laser injury by 1.6 ± 0.5-, 2.9 ± 0.7-, and 2.9 ± 0.5-fold compared with the control. There was a significant difference between wild-type mice and LOX-1-deficient mice with the induction of pro-MMP-2 at 3, 5, 9, and 21 days after laser injury ($P = 0.04$, $P = 0.012$, $P = 0.05$, and $P = 0.044$, respectively), and there was a significant difference

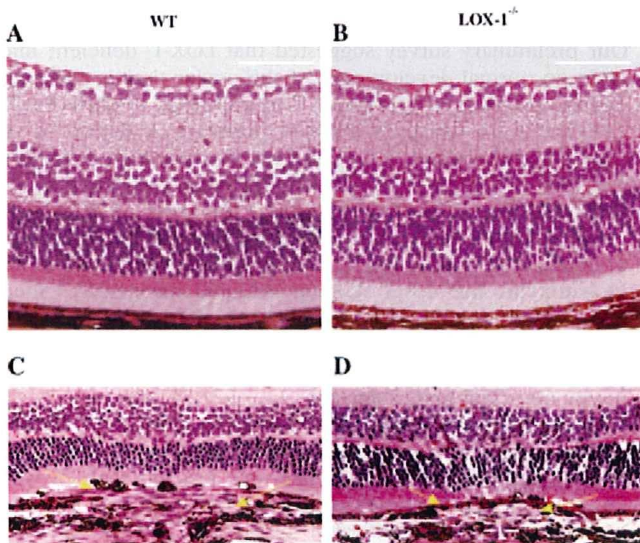
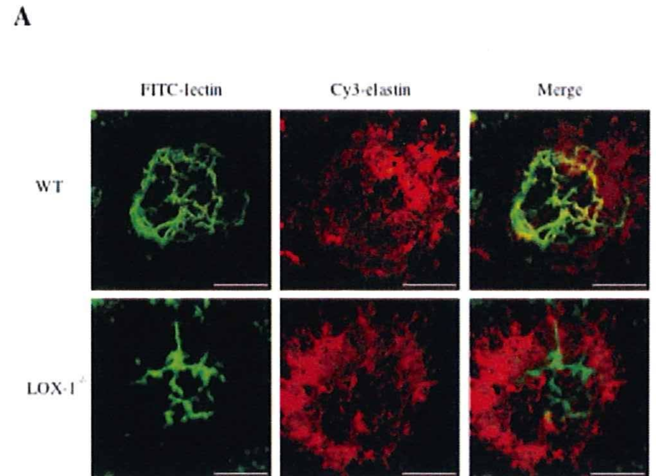


FIGURE 2. Histologic CNV sections in wild-type and LOX-1-deficient mice. Representative light micrographs of hematoxylin and eosin-stained sections of posterior segment in wild-type (**A**) and LOX-1-deficient (**B**) without laser injury and the middle of the CNV lesions in wild-type (**C**) and LOX-1-deficient (**D**) mice 14 days after laser injury. (**C**, **D**, *arrows*) Proliferative membranes. Scale bars, 50 μm.

between wild-type mice and LOX-1-deficient mice with the induction of MMP-2 at 3, 5, and 9 days after laser injury (P = 0.021, P = 0.037, and P = 0.039, respectively).

MCP-1 and VEGF Protein Expression

To identify and quantify MCP-1 and VEGF protein expression, we carried out ELISA of ocular tissue samples from eyes after laser injury (Fig. 6). The expression of MCP-1 protein was induced within 3 days of laser treatment in wild-type eyes and then gradually returned to basal levels by Western blot analysis (data not shown). Therefore, we detected MCP-1 protein expression 3 days after laser injury by ELISA. MCP-1 was not detected in wild-type mice or LOX-1-deficient mice without laser injury, and it increased in wild-type mice 3 days after laser injury (110.7 ± 38.1 pg/mg protein). MCP-1 protein expression in LOX-1-deficient mice (63.4 ± 14.2 pg/mg protein) was significantly decreased compared with wild-type mice (P = 0.014). Previous reports demonstrated that VEGF concentrations peaked at 3 days after laser injury.[22,23] Accordingly, we examined VEGF protein expression 3 days after laser injury by ELISA. Without laser injury, VEGF protein expression was not significantly different between wild-type mice (16.1 ± 5.2 pg/mg protein) and LOX-1-deficient mice (20.2 ± 11.3 pg/mg protein). Although VEGF protein expression was clearly increased in wild-type mice (121.3 ± 18.2 pg/mg protein) 3 days after laser injury, the increasing level was markedly decreased in LOX-1-deficient mice (93.4 ± 13.1 pg/mg protein; P = 0.032).



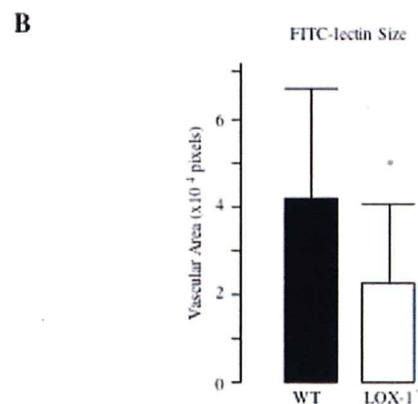
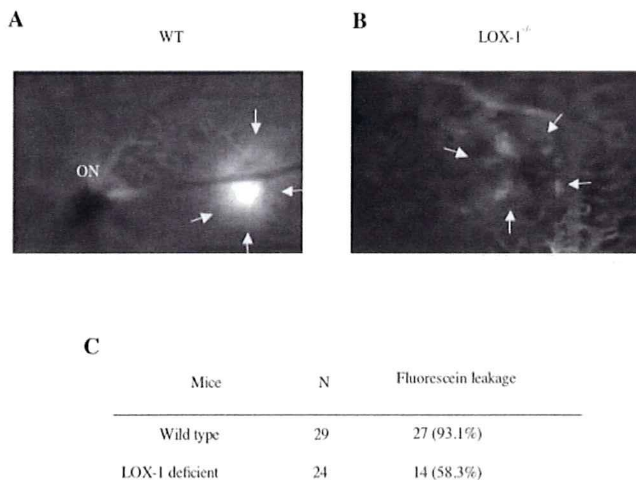


FIGURE 4. (**A**) Representative micrographs of CNV lesions 14 days after laser injury, stained with fluorescein isothiocyanate (FITC)-lectin (*left*) and Cy3-labeled elastin (*middle*) in wild-type mice (*upper*) and LOX-1-deficient mice (*lower*). Scale bars, 50 μm. (**B**) Computer image analysis revealed a significantly smaller CNV area in LOX-1-deficient mice (four eyes) than in wild-type mice (four eyes). *Asterisks*: statistically significant differences (P = 0.011). Absolute values were as follows: n = 12 and mean ± SD = 4.27 ± 2.58 × 10^4 pixels for wild-type mice; n = 12 and mean ± SD = 2.46 ± 1.63 × 10^4 pixels for LOX-1-deficient mice.



Mice	N	Fluorescein leakage
Wild type	29	27 (93.1%)
LOX-1 deficient	24	14 (58.3%)

FIGURE 3. Representative images of the late stage of fluorescein angiography 14 days after laser injury in wild-type (**A**) and LOX-1-deficient (**B**) mice. (**C**) Fluorescein leakage-spot data in wild-type (eight eyes) and LOX-1-deficient (six eyes) mice. *Arrows*: leakage points. ON, optic nerve.

DISCUSSION

Numerous factors, including oxidative stress, inflammatory reactions (such as complement activation), upregulated chemokines, and remodeling in the extracellular matrices, are involved in the pathogenesis of CNV in eyes with AMD.[25-28] Earlier studies have demonstrated that the drusen and CNVs of

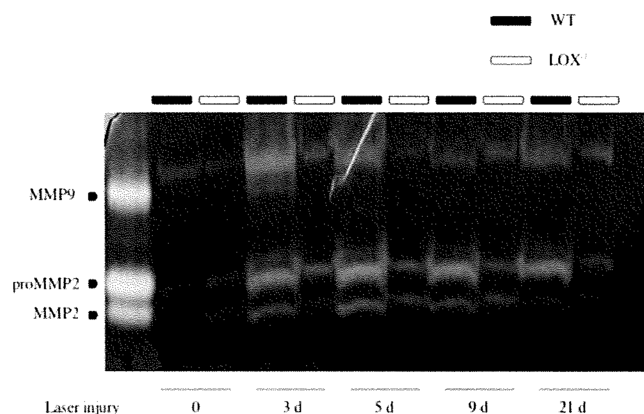


FIGURE 5. Gelatin zymography of wild-type (*black bars*) and LOX-1-deficient (*white bars*) mouse eyes after laser injury. Increased levels of pro-MMP-2, pro-MMP-9, and activated MMP-2 were evident on days 3, 5, and 9 after laser injury in wild-type, but not LOX-1-deficient, mice.

patients with AMD share similar features with atherosclerotic processes,[6,29] and it has recently been hypothesized that the pathogenesis of AMD is similar to that of atherosclerosis.[5,6]

Among these common features, the involvement of ox-LDL and its specific receptors is of particular interest because of their possible critical roles in the pathogenesis of atherosclerosis.[7] During the first stage of atherosclerosis, scavenger receptor-mediated recognition of ox-LDL by macrophages and endothelial cells leads to the formation of foam cells.

LOX-1, which is the major receptor for ox-LDL in the endothelial cells of large arteries,[30,31] was initially identified in bovine aortic endothelial cells[14] and is a type 2 transmembrane protein with a C-type lectinlike extracellular domain. Indeed, LOX-1 deletion in LDL receptor (LDLR)-deficient mice leads to reduced atherogenesis formation in vivo.[15] Previously, we showed that LOX-1 is expressed in surgically obtained CNV specimens.[16] From this observation, we hypothesized that LOX-1 is involved in the pathogenesis of CNV formation and that elucidation of the molecular basis of its contribution to angiogenic lesions in the ocular fundus might lead to novel therapeutic concepts.

In the present study, real-time RT-PCR revealed the upregulated expression of LOX-1 mRNA in the acute phase after laser injury to the retina. We were also able to detect the upregulated expression of LOX-1 protein by laser injury in Western blot analysis, but we were unable to localize the upregulated expression of LOX-1 in immunohistochemistry. Previous investigations have suggested that a number of factors can induce LOX-1 expression under inflammatory conditions. For example, LOX-1 can be immediately induced by proinflammatory stimulants, such as the presence of oxidant species,[32] cytokines,[33] and shear stress,[34] suggesting that it is an immediate-early gene. Our results are in accordance with these findings because laser injury causes the induction of proinflammatory cytokines in the injured region.

Upregulated LOX-1 expression has also been indicated in inflammatory changes of the vascular endothelium through the generation of superoxide,[35] the reduction of nitric oxide,[36] the induction of MCP-1,[18] and the promotion of leukocyte adhesion.[37] We previously observed that the inhibition of LOX-1-mediated effects greatly decreased the inflammatory reaction of animal eyes by reducing the adhesion between circulating leukocytes and retinal vascular endothelial cells.[37] Therefore, we concluded that LOX-1 is a deteriorating factor in retinal and/or choroid inflammatory reactions as part of a cascade. However, previous studies (including our own) of clinical human CNV samples have demonstrated LOX-1 expression in vascular endothelial cells or some macrophages, suggesting that its prolonged expression contributes to the development of inflammatory reactions in the angiogenic lesions of elderly patients.

The present study revealed that the formation of CNV after laser injury is inhibited in LOX-1-deficient mice. This was confirmed by fluorescein angiography and lectin staining and implied that LOX-1 plays an important role in the pathogenesis of CNV lesions. Given that LOX-1 is involved in many inflammatory reactions of diseased tissues, its reduced expression leading to a deficiency of inflammation would inhibit the formation of CNV after laser injury. In agreement with this theory, we previously showed that inhibition of LOX-1-mediated effects elicited anti-inflammatory activity in inflammatory lesions of the eye. Of course, further examination of the neovascular response with the use of antiserum to block the function of LOX-1 will be required to confirm our hypothesis. Our present study of the LOX-1 effect on MMP and MCP-1 expression further investigated this hypothesis, with particular emphasis on proinflammatory cytokines and related enzymes.

MMPs, especially MMP-2 and MMP-9, are important enzymes for vascular remodeling in many disorders, and their levels are increased in human CNVs with exudative AMD. Mice that were doubly deficient for MMP-2 and MMP-9 demonstrated attenuated CNV in terms of incidence and severity compared with single gene-deficient mice or corresponding wild-type controls. From these findings, it was deduced that MMP-2 and MMP-9 might cooperate in the development of AMD[10] It has also been shown that reduced CNV formation occurs in MMP-2-deficient mice.[38] Our results suggest that a deletion of LOX-1 inhibits the induction pro-MMP-2 and pro-MMP-9 and the activation of MMP-2 after laser injury.

Some previous studies have found that MMP-9 microsatellite polymorphisms are associated with susceptibility to the exudative form of AMD,[39] whereas others have described elevated levels of MMP-9 in the plasma of patients with AMD.[40] Hence, there is increasing evidence for the critical involvement of

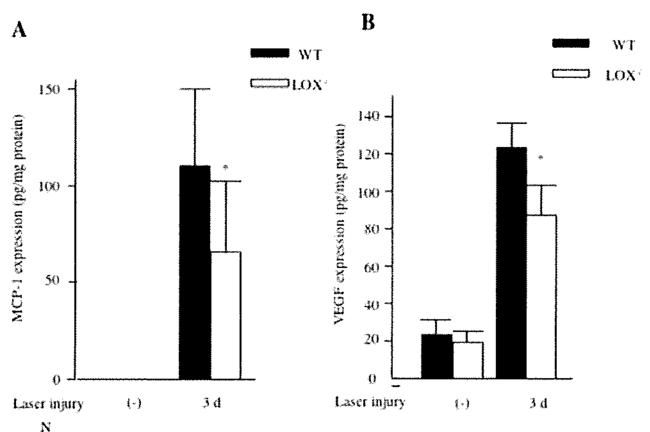


FIGURE 6. Assessment of MCP-1 and VEGF levels in eyes from wild-type (*black bars*) and LOX-1-deficient (*white bars*) mice after laser injury. MCP-1 (**A**) and VEGF (**B**) protein expression were measured by ELISA in eyes from wild-type (*black bars*) and LOX-1-deficient (*white bars*) mice without laser injury and 3 days after laser injury. MCP-1 protein expression was not detected in wild-type mice or LOX-1-deficient mice ($n = 5$ and $n = 4$, respectively). The concentrations of MCP-1 protein were 110.7 ± 38.1 in wild-type mice and 63.4 ± 14.2 in LOX-1-deficient mice ($n = 12$ and $n = 6$, respectively) 3 days after laser injury. The concentration of VEGF protein was 16.1 ± 5.2 in wild-type mice and 20.2 ± 11.3 in LOX-1-deficient mice without laser injury ($n = 5$ and $n = 4$, respectively). Three days after laser injury, the concentration of VEGF protein was 121.3 ± 18.2 in wild-type mice and 93.4 ± 13.1 in LOX-1-deficient mice ($n = 8$ and $n = 8$, respectively).

MMPs and the resultant remodeling of extracellular matrices in the pathogenesis of CNV. Our zymographic experiments indicated that the molecular mechanisms of LOX-1, in relation to CNV formation, might be elicited by the regulation of MMP-2 and MMP-9.

Activations of LOX-1 and MCP-1 were also demonstrated to be collectively involved in the early stages of atherosclerosis in hypertensive rats.[41] Our animal experiments revealed that the deletion of LOX-1 inhibited the expression of MCP-1 after laser injury. MCP-1 is well known to play as an important molecule in monocyte recruitment and angiogenic processes. Previous studies reported that MCP-1 is a key factor for the formation of CNV after laser injury.[42,43] Our finding suggested that the involvement of MCP-1 in CNV membranes has been demonstrated in clinical and experimental samples, in agreement with our findings.[13,43,44]

Using a specific antisense to human LOX-1 mRNA, the receptor has been shown to be a key factor in regulating the expression of MCP-1 and ox-LDL-mediated monocyte adhesion to vascular endothelial cells.[18] On the other hand, LOX-1-dependent redox signal pathways have been shown to promote the expression of VEGF induced by angiotensin II and the expression of MMPs induced by ox-LDL in endothelial cells.[17,45] These findings propose a hypothesis that the LOX-1-mediated redox signal pathway, including mitogen-activated protein kinase, is a crucial factor for angiogenesis mediated by MCP-1, VEGF, and MMPs. Our present study indicated that the activation of MMPs and the expression of MCP-1 and VEGF by laser injury were suppressed in LOX-1-deficient mice compared with wild-type mice. However, we could not examine the development of CNV at various time points and assay the expression of LOX-1, VEGF, MCP-1, and MMPs at the same time points in this study because we determined the time points based on previous reports[10,13,19,23] and the LOX-1-deficient mice were limited. Based on our finding, the relationships among LOX-1, MCP-1, VEGF, and MMPs were not clarified in the development of CNV. Therefore, further studies will be required to fully elucidate these relationships and the complicated network of AMD pathogenesis.

In conclusion, the present study shows that upregulated expression of LOX-1 can be induced by laser injury. Our results clearly indicate, for the first time, the involvement of LOX-1 and associated factors in the formation and deterioration of CNV. Although further studies will be needed to clarify the significance of LOX-1 in the pathogenesis of AMD, this work suggests that the inhibition of SRs could be a novel therapeutic modality for AMD.

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Advance Publication by J-STAGE

LOX-1
—— The Multifunctional Receptor Underlying Cardiovascular Dysfunction ——

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Oxidatively modified low-density lipoprotein (oxLDL) is implicated in the pathogenesis of atherosclerosis. Endothelial dysfunction is the initial change in the vascular wall that induces morphological changes for atheroma-formation. Lectin-like oxidized LDL receptor-1 (LOX-1) was identified as the receptor for oxLDL that was thought to be a major cause of endothelial dysfunction. LOX-1 has been demonstrated to contribute not only to endothelial dysfunction, but also to atherosclerotic-plaque formation, myocardial infarction and intimal thickening after balloon injury. Recent findings on the genetics of LOX-1 and the methodology to detect it and its ligands would further facilitate the examination of the receptor's pathophysiological contribution in atherosclerosis. Furthermore, LOX-1-related tools might open new gateways from diagnosis to therapeutics for cardiovascular diseases.

Key Words: Atherosclerosis; Endothelial dysfunction; LOX-1; Oxidized low-density lipoprotein

Atherosclerosis is characterized by the accumulation of lipids and fibrous elements in the arteries, and is the most important contributor to the growing burden of cardiovascular diseases. The major risk factors of atherosclerosis, such as hypertension, diabetes, smoking and free radicals, have been known to induce endothelial dysfunction.[1,2] Endothelial dysfunction, functional changes in endothelial cells, has been thought to precede morphological changes of atheroma.[1] Lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) was identified from endothelial cells as the molecule that induces endothelial dysfunction triggered by oxidized low-density lipoprotein (LDL).[3] LOX-1 is a 50 kDa type II transmembrane glycoprotein comprising 273 amino acids.[3] The protein contains a short N-terminal cytoplasmic domain, a single transmembrane domain and an extracellular domain comprising a neck domain followed by a C-terminal C-type lectin-like ligand-binding domain (**Figure 1**).[4] LOX-1 has been demonstrated to actively contribute to all stages of atherogenesis. LOX-1 is expressed not only in endothelial cells, but also in macrophages,[5] vascular smooth muscle cells[6] and platelets.[7] In vitro, the basal expression of LOX-1 in endothelial cells is limited; however, it can be rapidly induced by proinflammatory, pro-oxidative and mechanical stimuli.[8–14] In vivo, the basal expression of LOX-1 is also low, but can be enhanced by several pathological conditions, including hypertension,[15] diabetes mellitus,[16] hyperlipidemia[17] and chronic renal failure.[18] Here, we aim to briefly review the roles of LOX-1 in various diseases to understand the vascular, as well as myocardial, LOX-1 function in basic and clinical medicine.

LOX-1 Signaling

Oxidative modification of LDL (oxLDL) has been known to increase its atherogenecity.[19] Several reports have revealed that elevated plasma levels of oxLDL are associated with coronary artery diseases (CAD).[20] The plasma levels of oxLDL are related to the presence of angiographically detected complex and thrombotic lesion morphology in patients with unstable angina.[21] LOX-1 binding to oxLDL enhances nitric oxide (NO) catabolism as a result of superoxide generation, and decreases NO release via attenuated endothelial NO synthase (eNOS) activity. LOX-1 has been recently shown to form a complex with MT1-MMP under a basal condition.[22] When oxLDL binds to LOX-1 it induces rapid RhoA and Rac1 activation via MT1-MMP, which results in NADPH oxidase activation and eNOS downregulation.[22] The imbalance of NO and oxidative stress resulting from the binding of oxLDL to LOX-1 causes oxLDL-induced endothelial dysfunction leading to atherosclerosis.

In addition to the Rho and Rac pathways, the following signal transduction pathways have been reported to be activated via LOX-1: p38 mitogen-activated protein kinase C (MAPK),[23,24] p44/42MAPK,[11] protein kinase C,[25] protein kinase B,[26] ERK1/2,[27] protein tyrosine kinase[28] and NF-κB. Among them, LOX-1-mediated NF-κB activation by oxLDL is crucial for increasing the expressions of the following adhesion molecules: E- and P-selectins, intracellular adhe-

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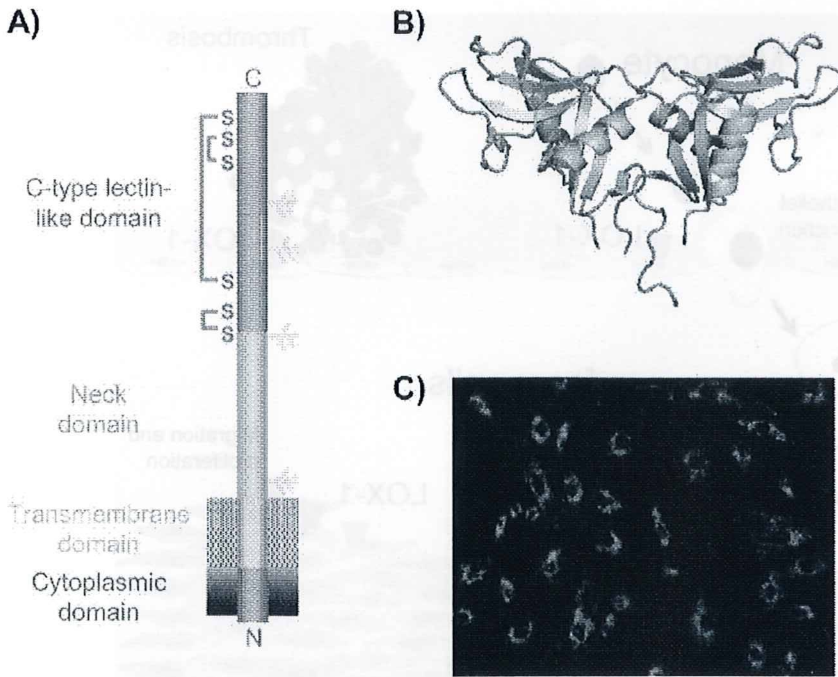

Figure 1. (A) Schematic model of the structure of human lectin-like oxidized LDL receptor-1 (LOX-1). LOX-1 has 4 domains: cytoplasmic, transmembrane, neck and C-lectin-like. (B) LOX-1 forms homodimers under physiological conditions. (C) DiI-oxLDL uptake in human umbilical vein endothelial cells (HUVECs).

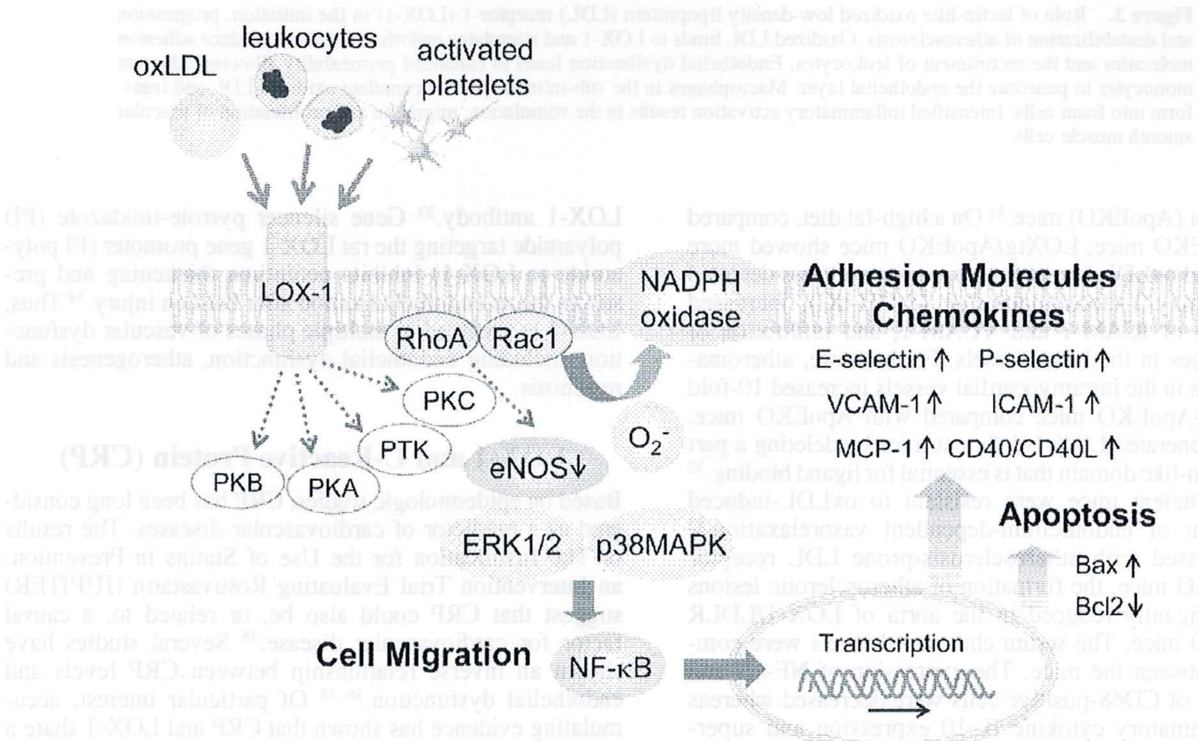

Figure 2. Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) signaling pathway. MAPK, mitogen-activated protein kinase C; PKC, protein kinase C; PKB, protein kinase B; PTK, protein tyrosine kinase; eNOS, endothelial nitric oxide synthase; ICAM-1, intracellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; MCP-1, monocyte chemotactic protein-1.

sion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemotactic protein-1 (MCP-1),[25,29,30] which brings proinflammatory changes to the vessel wall. Additionally, LOX-1 activation changes endothelial cells and smooth muscle cells prone to apoptosis by increasing the Bcl-2-associated X protein (Bax)/Bcl-2 ratio (**Figure 2**).[6]

LOX-1 and the Vascular System

Atherosclerosis is characterized by chronic local inflammation of the vascular wall.[1] To date, multiple lines of evidence have implicated LOX-1 in atherosclerosis (**Figure 3**). We created mice over-expressing bovine LOX-1 (LOXtg) and crossed them with atherosclerosis-prone apolipoprotein

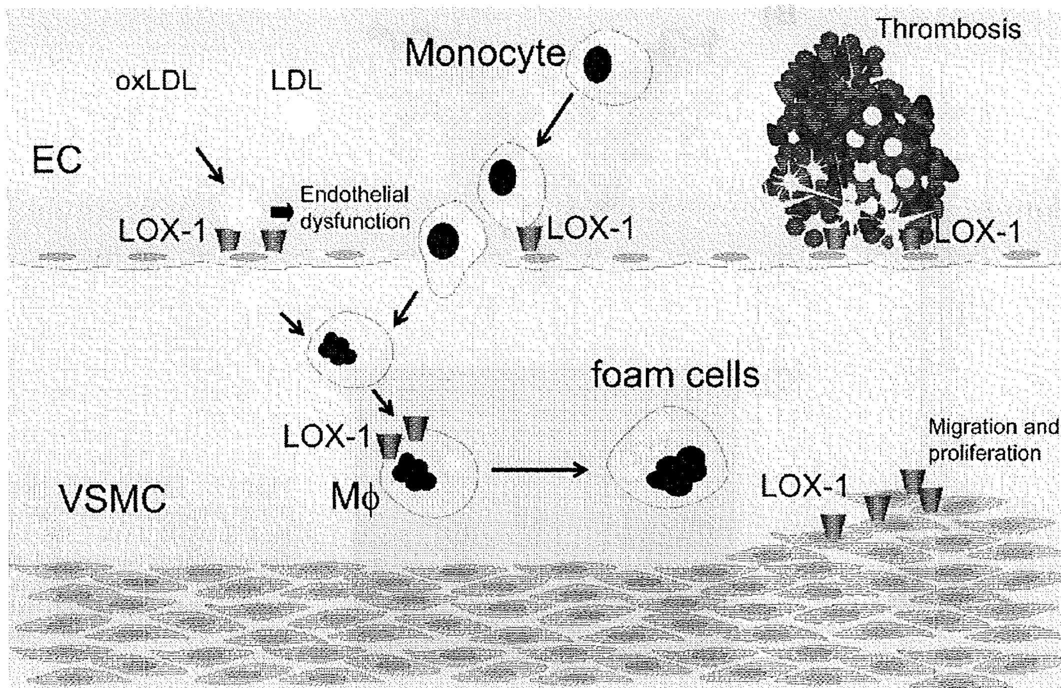

Figure 3. Role of lectin-like oxidized low-density lipoprotein (LDL) receptor-1 (LOX-1) in the initiation, progression and destabilization of atherosclerosis. Oxidized LDL binds to LOX-1 and stimulates endothelial cells to produce adhesion molecules and the recruitment of leukocytes. Endothelial dysfunction leads to enhanced permeability allowing adherent monocytes to penetrate the endothelial layer. Macrophages in the sub-intimal space accumulate oxidized LDL and transform into foam cells. Intensified inflammatory activation results in the stimulation, migration and proliferation of vascular smooth muscle cells.

E knockout (ApoEKO) mice.[31] On a high-fat diet, compared with ApoEKO mice, LOXtg/ApoEKO mice showed more pronounced oxLDL accumulation, oxidative stress detected by 8-hydroxy-2'-deoxyguanosine (8-OH-dG), increased expression of ICAM-1 and VCAM-1, and infiltration of macrophages in the heart vessels. Furthermore, atheroma-like lesions in the intramyocardial vessels increased 10-fold in LOXtg/ApoEKO mice compared with ApoEKO mice. We also generated LOX-1-deficient mice by deleting a part of the lectin-like domain that is essential for ligand binding.[32] LOX-1-deficient mice were resistant to oxLDL-induced impairment of endothelium-dependent vasorelaxation.[32] When crossed with atherosclerosis-prone LDL receptor (LDLR) KO mice, the formation of atherosclerotic lesions was significantly reduced in the aorta of LOX-1/LDLR double KO mice. The serum cholesterol levels were comparable between the mice. The expression of NF-κB and infiltration of CD68-positive cells were decreased whereas anti-inflammatory cytokine IL-10 expression and superoxide dismutase activity were increased in the double KO mice.[32] LOX-1 gene deletion also resulted in less aterial collagen accumulation in LDL KO mice. These gain-of-function and loss-of-function approaches clearly demonstrate that LOX-1 plays a role in the development of atherosclerosis (**Figure 4**).

LOX-1 is likely to mediate inappropriate arterial remodeling, which is one of the causes of atherosclerosis and restenosis. In a balloon-injured carotid artery, strong LOX-1 expression was observed at first in injured medial smooth muscle cells, then in proliferating intimal smooth muscle cells, and finally in the regenerated endothelial cells. The reactive intimal thickening, ROS generation and leukocyte infiltration were attenuated by the administration of anti-LOX-1 antibody.[33] Gene silencer pyrrole-imidazole (PI) polyamide targeting the rat LOX-1 gene promoter (PI polyamide to LOX-1) inhibits neointima thickening and preserves the re-endothelialization after balloon injury.[34] Thus, LOX-1 is involved in multiple phases of vascular dysfunction, including endothelial dysfunction, atherogenesis and restenosis.

LOX-1 and C-Reactive Protein (CRP)

Based on epidemiologic studies, CRP has been long considered as a predictor of cardiovascular diseases. The results of The Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) suggest that CRP could also be, or related to, a causal factor for cardiovascular disease.[35] Several studies have shown an inverse relationship between CRP levels and endothelial dysfunction.[36–38] Of particular interest, accumulating evidence has shown that CRP and LOX-1 share a range of biological functions. LOX-1 mRNA and proteins are induced by CRP, resulting in increased monocyte adhesion to endothelial cells and oxLDL uptake.[9] Furthermore, it has been shown that CRP in vivo impairs endothelial vasoreactivity.[39,40] It has been shown that CRP-LOX-1 binding enhances vascular permeability in vivo in SHR-SP rats.[41] LOX-1 exhibits binding activity for multiple ligands, apoptic cells,[42] bacteria[43] and phoshatidylserine,[44] all of which are also recognized by CRP.[45] CRP binding to LOX-1 enhances the binding affinity of oxLDL to LOX-1.[46] CRP induces the expressions of ICAM-1, VCAM-1, E-selectin and chemokine MCP-1 as well as the activation of RhoA and p38 (MAPK).[47,48] CRP-mediated expressions of ICAM-1 and VCAM-1 genes are inhibited by LOX-1 knockdown.[46]

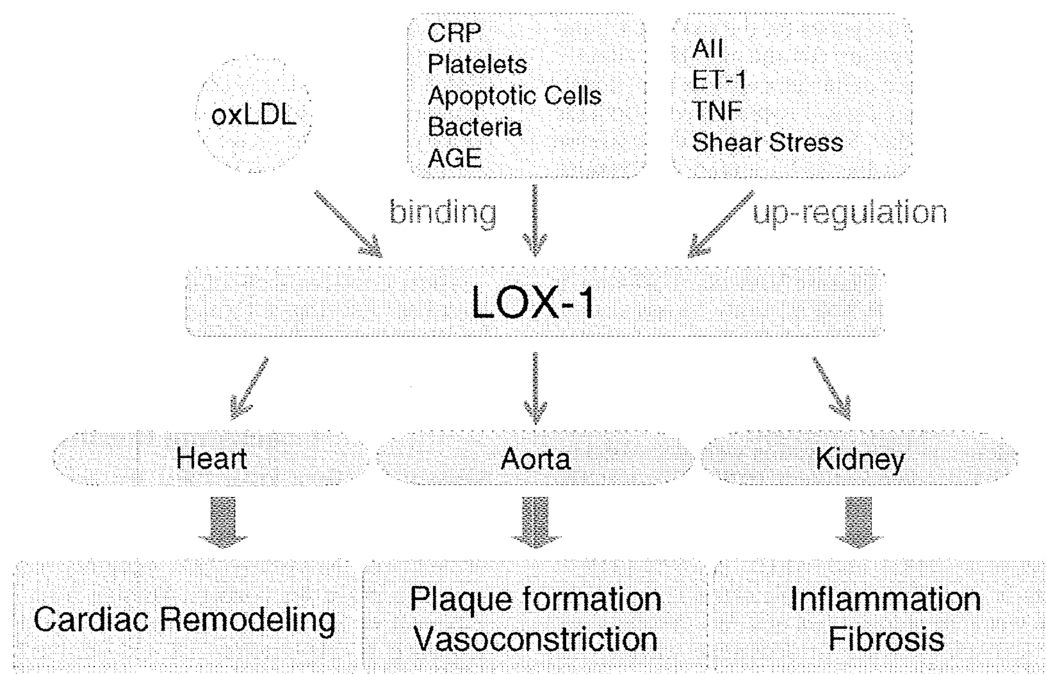

Figure 4. LOX-1 is bound and/or upregulated by the various factors indicate. The functions of LOX-1 contribute to cardiac remodeling, plaque formation, vasoconstriction and renal injury. The coordination of these triggers the onset of multiple organ damage.

These lines of evidence support the hypothesis that the CRP interaction with LOX-1 might play a role in atherogenetic inflammation, which is relevant to endothelial dysfunction.

LOX-1 and Myocardial Infarction (MI)

LOX-1 is also readily detectable in cardiomyocytes under prooxidative conditions (**Figure 4**). In vitro, a blockade of LOX-1 inhibits NF-κB activations and diminishes apoptosis, which suggests that LOX-1 plays a role in oxidative stress in cardiomyocytes.[49,50] In vivo, the expression of LOX-1 in cardiomyocytes is prominently induced by ischemia–reperfusion.[49,50] Treatment with anti-LOX-1 antibody prevents cardiac remodeling in a rat model of myocardial ischemia–reperfusion. It also reduces the size of myocardial infarct and improves left ventricular function by inhibiting apoptosis and lipid oxidation in cardiomyocytes.[49,50] Deletion of the LOX-1 gene in mice reduces myocardial remodeling following ischemia–reperfusion, attenuated NADPH oxidase expression and decreased collagen deposition.[51] LOX-1 KO mice further showed a significantly smaller decline in –dP/dt max and a smaller increase in left ventricle end-diastolic pressure after ischemia–reperfusion compared with the control group.[51]

LOX-1 and Kidney Injury

Chronic kidney disease is a risk factor for cardiovascular diseases and the enhanced progression of renal dysfunction has been reported in patients with obesity and hypertension. Several reports showed the association between LOX-1 and renal dysfunction (**Figure 4**). LOX-1 expression was increased in the renal tubules of obese–diabetic Zuker Spontaneous hypertensive heart failure (ZS) rats.[52] The anti-LOX-1 antibody injection reduced renal inflammation and fibrosis in ZS rats.[53] Kidneys from ZS rats treated with anti-LOX-1 antibody lacked neutrophil infiltration, whereas kidneys from untreated and normal IgG-injected ZS rats had abundant clusters of neutrophils along the capillary lumens. Although anti-LOX-1 antibody did not prevent albuminuria, it increased glomerular vascular endothelial growth factor levels to help preserve renal microvascular beds.[53] It was also shown that the expression of the AT1 receptor in LOX-1 KO mice was lower than wild-type mice both before and after infusion of angiotensin II (Ang II).[54] Furthermore, Ang II-induced, but not norepinephrine-induced, blood pressure increase and renal injury were attenuated in LOX-1 KO mice compared with wild-type mice.[54] LOX-1 KO mice infused with Ang II exhibited less glomerulosclerosis, arteriolar sclerosis and tubulointerstitial damage. These findings highlight the fact that the Ang II-AT1R-LOX-1 loop is an important regulator of blood pressure and renal injury. Thus, LOX-1 plays a pathological role in the heart and kidney as well as in blood vessels.

Genetics

The human LOX-1 gene spans more than 7,000 base pairs and consists of 6 exons and 5 introns. LOX-1 is within a C-type lectin gene cluster on chromosome 12p12-13.[4] Several groups have analyzed the association of LOX-1 gene polymorphisms with CAD. The 3'-untranslated region (UTR) (T allele), a C-to-T change 188 nucleotides from the stop codon (+188C-T), was associated with a higher risk of acute MI.[55] Furthermore, 7 different single nucleotide polymorphisms (SNPs), 6 of them located within introns 4, 5 and 3' UTR comprised in a linkage disequilibrium block, exhibited a significant association with an elevated risk of developing MI.[56] The SNPs give rise to a splicing variant lacking exon 5, named LOXIN, which lacks two-thirds of the ligand binding domain of LOX-1. The LOX-1/LOXIN mRNA ratio is 33% higher in monocyte-derived macro-

phages of the subjects homozygous for the risk allele compared with those homozygous for the non-risk allele.[57] LOXIN probably works as a dominant negative form dimerizing with the native form of LOX-1 to protect cells from the damage by oxLDL. Another SNP leading to the missense mutation of Lys to Asn at the 167th amino acid residue (K167N) was identified and reported to be associated with an increased risk of MI.[58] However, this association could not be replicated in a later study using a larger population. In the Atherosclerotic Disease VAscular FuNction & GenetiC Epidemiology (ADVANCE) study, an association of K167N with a decreased risk of CAD was observed, whereas in the Atherosclerosis Risk in Communities (ARIC) study using a larger population, the association was reported to be insignificant.[59] Recently, biochemical analysis showed that oxLDL binding and ERK activation were decreased by 30% in 167N-LOX-1 compared with 167K-LOX-1.[60] The structural analysis suggests that the reduced binding of oxLDL could be ascribed to the decrease of the electrostatic interaction between LOX-1 and oxLDL in 167N-LOX-1. Thus, it might be plausible that people carrying the 167N-LOX-1 allele would be at lower risk of CAD. Presently, we do not have enough evidence to determine whether the SNPs in the LOX-1 gene are useful to assess the risk and prognosis of CAD.

LOX-1 as a Clinical Tool

Soluble LOX-1

A number of membrane proteins are cleaved into soluble molecules by proteolysis at the membrane-proximal site of the extracellular domain, which is known as ectodomain shedding. LOX-1 is also released in a soluble form from the cell surface into culture media.[61] There are 2 cleavage sites that have been identified to yield soluble LOX-1 (sLOX-1; ie, Arg^{86}-Ser^{87} and Lys^{89}-Ser^{90} bonds of bovine LOX-1).[61] A member of the A Disintegrin And Metalloproteinase (ADAM) family, ADAM10, contributes to the proteolysis of LOX-1.[62] Recently, it has been reported that the serum sLOX-1 level is specifically elevated in acute coronary syndrome.[63] Peak values of sLOX-1 are observed earlier than those of Troponin T in acute coronary syndrome. Furthermore, the sensitivity and specificity of sLOX-1 in acute coronary syndrome are significantly better than high sensitivity CRP.

LOX-1 Ligand Assay

A receptor-based assay to determine the levels of modified LDL as LOX-1 ligands has been reported.[64–66] Although clinical assessment is yet to be carried out, the LOX-1 ligand level is elevated in hyperlipidemic animals in association with the extent of atheroma-formation. The circulating amount of oxidation-related moiety on LDL has been shown to be effective in predicting or diagnosing metabolic syndrome and cardiovascular diseases.[67–69] Considering that oxLDL interacts with cells via its receptors, the receptor binding activity is likely to reflect the biological activity of oxLDL better than the absolute amount of oxidatively modified moiety.

Imaging

Taking an advantage of the relatively selective expression of LOX-1 in atheroma, the methodology to obtain images of atheroma utilizing the anti-LOX-1 antibody was reported.[70] ^{99m}Tc-labeled anti-LOX-1 antibody administered into MI-prone Watanabe heritable hyperlipidemic rabbits accumulated in each aortic segment, which was a significantly higher level than that in control rabbits. High density accumulation is also observed at collagen-rich and neointimal lesions. The macrophage density and vulnerability index correlated with ^{99m}Tc-LOX-antibody accumulation, but not with ^{99m}Tc-IgG accumulation. This nuclear imaging technique might be useful for the diagnosis of plaque vulnerability.

Thus, effective tools utilizing LOX-1 and its antibody have been developed. Clinical application of LOX-1 would be diagnostic rather than therapeutic, while it warrants further investigations.

Concluding Remarks

Since it has been known that the modification of LDL increases its atherogenicity, people have been seeking what underlies "oxidation hypothesis". Identification and series of studies on the oxidized LDL receptors, including LOX-1, seems to prove the concept. Starting from epidemiology, a branch of the study of atherosclerosis proceeded in a sequence of cholesterol-LDL-oxLDL-oxLDL receptors. LOX-1 showed unexpectedly diverse ligand specificity with versatile functions, which points to a new avenue of research.

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