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Review

Oxidized low-density lipoprotein receptor, LOX-1, on the endothelial cell – The receptor structure and functions of LOX-1 in atherogenesis

Shin-ichi Tate[1,2]

[1] Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8526, JAPAN

[2] PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama, JAPAN

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Oxidized LDL and its receptors in atherogenesis

Atherosclerosis is responsible for most of deaths, which includes coronary heart disease and interference with blood supply to the brain, thus resulting in apoplectic stroke. Indeed, deaths caused by atherosclerosis are accounting for the deaths by cancer, the most common cause of death at present. Atherosclerosis also causes other illness by reducing the flow of blood in other arteries, including the kidneys, legs, and intestines; the reduced blood flow in those organs should be the troubles in keeping a person's quality of life. In the atherosclerotic arteries, plaques (raised patches) are formed. The plaques contain low-density lipoproteins, decaying smooth muscle cells, fibrous tissues, clumps of blood platelets, and cholesterol. The formation of the plaques makes the inner lining of the arteries narrower. The number of the plaques increases with age, causing loss of the smooth lining of the blood vessels and encouraging the formation of thrombi.

Many causes of atherosclerosis are pointed, including hypertension (high blood pressure), smoking, diabetes, obesity, cholesteremia and family history of heart disease. Oxidative stress is recognized as the central cause for atherosclerosis (1). Oxidative stress modifies low density lipoprotein (LDL), thus resulting in the production of oxidized LDL (OxLDL) in the blood flow. OxLDL is recognized as a key molecule in the development of atherosclerosis (2-5). In the intima of blood vessel, OxLDL is up taken by macrophages that are converted from monocytes. The macrophages having incorporated excess formation. The OxLDL also induces apoptosis in endothelial cells, which thus causes plaque rupture (7,8). In the development of atherosclerosis, the foam cell formation has been well studied to identify several types of OxLDL receptors, so called scavenger receptors, on the macrophages (9-11). Sawamura and co-workers have found a novel type of OxLDL receptor on endothelial cells, which was named lectin-like oxidized LDL receptor 1 (LOX-1) (12). In contrast to the OxLDL receptors on the macrophages, where various types of OxLDL receptors exist, LOX-1 is the single class of OxLDL receptor on human coronary artery endothelial cells (4,13,14). LOX-1 mediates OxLDL uptake into the endothelial cells. The process leads to endothelial cell dysfunction, which causes a series of events in the early stage of atherogenesis, which includes the expression a variety of genes such as endothelial nitric oxide synthetase (eNOS), cyclo-oxygenase, growth factor and monocyte chemoattractant protein-1 (MCP-1), and also LOX-1 (2,10,15). The up-regulated gene expression in endothelial cells induces a cascade of process of the early step in atherogenesis, which accompanies with the inflammatory cell migration to the site where the activated endothelial cells exist. The OxLDL mediated apoptosis in endothelial cells and the process was shown to be associated with up-regulation of LOX-1 (7,8). As descried, LOX-1 is recognized as the key receptor, which is engaged from the initial to the end stage in the atherogenesis (Fig.1).

In spite of the pathological importance of LOX-1, a lack of structural information for the receptor prohibited an in depth analysis of the LOX-1 – OxLDL interaction. We recently reported

Correspondence to:
E-mail: tate@hiroshima-u.c.jp

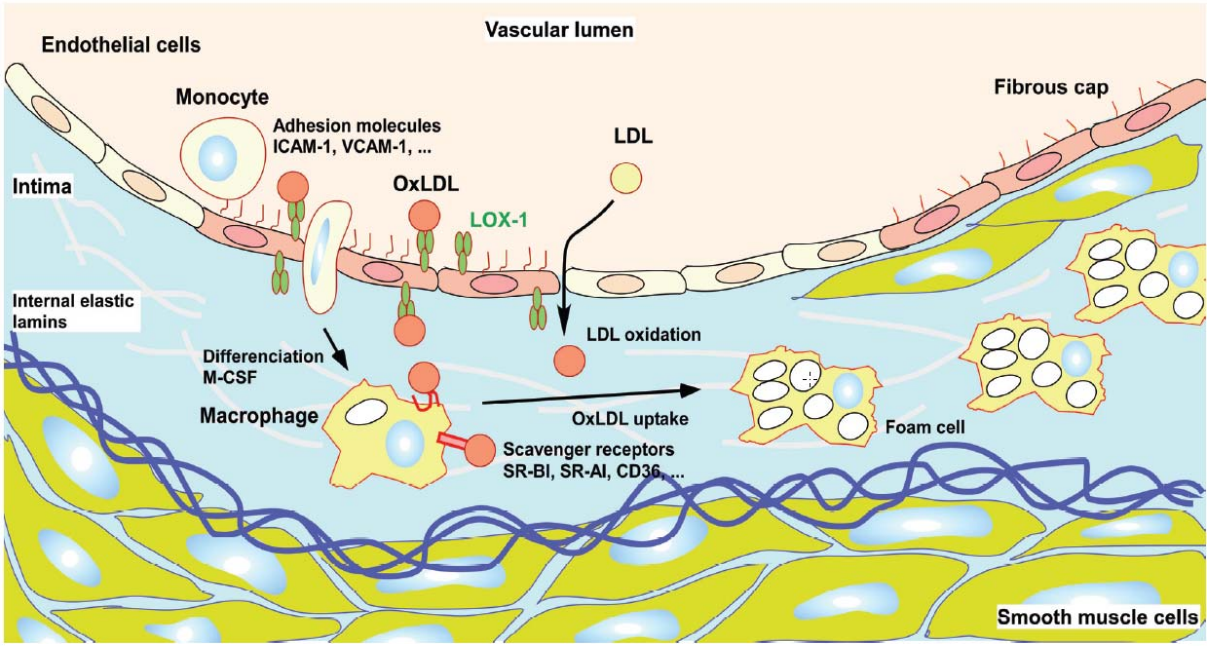

Fig. 1: Schematic illustration of the initial events leading to the development of a fatty streak. OxLDL in the blood flow or the generated in the intima interact with LOX-1 on the endothelial cell surface. LOX-1 binding to OxLDL causes endothelial dysfunction, colored in red. The endothelial dysfunction facilitates monocyte adhesion to the endothelial cells and its migration into the subendothelial space, where the monocyte is convereted into a macrophage. Uptake of OxLDL *via* scavenger receptors on the macrophage leads to foam cell formation. Foam cells integrate into the limited part in the vessel wall to form a fatty streak.

the crystal structure LOX-1 (16); working independently, Park et al. also reported the structure coincidently (17). The structure of LOX-1, in combination with various biochemical analyses on its interaction with OxLDL, has provided us fruitful information on the recognition mode of LOX-1 to OxLDL. In this review article, I summarize the structural properties and their relations to the function of LOX-1 (18).

A novel OxLDL receptor, LOX-1

OxLDL receptors on the macrophages have been mainly focused. Those identified OxLDL receptors, also called as scavenger receptors, include SR-AI/II, MARCO, CD36, SR-BI, CD68 and SREC (9,10). These classic scavenger receptors are absent or present in very small amounts on the endothelial cell surface. In contrast to the scavenger receptors, LOX-1 is a novel type of OxLDL receptor that is primarily found on endothelial cell (12). The classical scavenger receptors show a variety of structural architectures. Actually, the identified scavenger receptors show different protein architectures to each other. LOX-1 is also different in the architecture from those of the above scavenger receptors. LOX-1 is classified as a C-type lectin-like protein, with a type II orientation, which consists of four domains (Fig. 2): a short cytoplasmic domain, single transmembrane domain, stalk region (also called the NECK domain) and the C-type lectin-like domain (CTLD). The CTLD is shown to the OxLDL binding domain from the preceding studies using LOX-1 mutants.

We have successfully crystallized two forms of LOX-1 extracellular domain (16,19); one contains only the CTLD domain (residues 143-273) and the other consists of a short NECK and CTLD domains (residues 129-273),

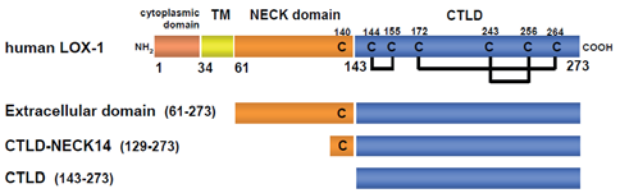

Fig. 2: The domain architecture of human LOX1. Two forms extracellular fragments, CTLD-NECK14 consisting of the residues 129-273 and CTLD comprising the residues 143-273, were successfully crystallized.

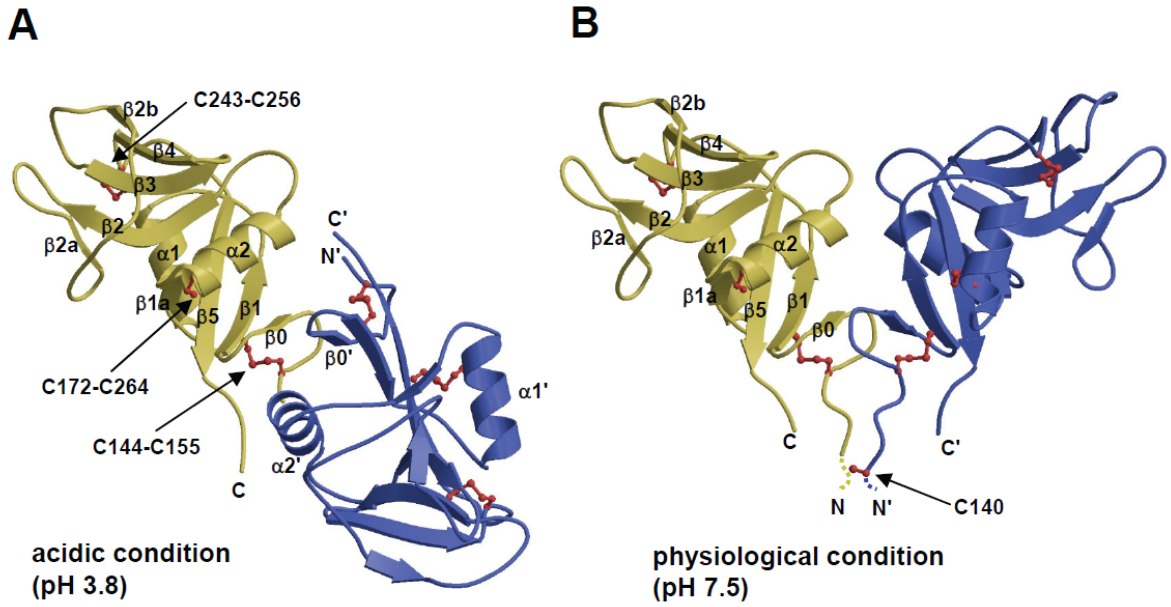

Fig. 3: Two crystal forms of LOX-1 for the extracellular fragments.. (A) Structure of the crystallographic dimer of LOX-1 CTLD (143-273). (B) Crystal structure of LOX-1 CTLD-NECK14 (129-273) that is linked by the inter-chain disulfide bond at C140.

CTLD-NECK14 (Fig. 2). Each fragment was engineered for expression in *E.coli*. Because each recombinant protein was obtained in insoluble fractions in bacterial cells, the proteins had to be subjected to *in vitro* refolding (19). In the present work, we successfully refolded the proteins, which contain three disulfide bonds in CTLD, by using "oxide shuffling reagent" that comprises a mixture of reduced and oxidized glutathione (GSH/GSSH mixture) (19). The LOX-1 fragment comprising only CTLD (143-273) was crystallized in acidic condition, and the other containing CTLD with short NECK, CTLD-NECK14 (129-273), was crystallized under physiological condition, pH 7.5. The crystal structures for these LOX-1 fragments show that they form homodimers in the crystalline state (Fig. 3). The domain arrangements, however, are different between these two crystal forms. The domain arrangement for CTLD-NECK14 seemed fixed by the inter-chain disulfide bond at C140, which residue in not in the CTLD fragment (143-273). It was shown that human LOX-1 exists as a disulfide-linked homodimer on the cell surface (20). The crystal structure of CTLD-NECK14, therefore, may represent the naturally occurring form of the LOX-1 extracellular domain. Although the significance of the altered domain arrangement for the CTLD fragment is not clear, this observation demonstrates that the dimer interface is fragile at least in acidic condition. We favor to think that this intrinsic instability of the CTLD dimer at acidic condition is related to OxLDL releasing from LOX-1 in the endosome; the interior pH of the endosome is measured to be acidic, around pH 5, thus the internalized LOX-1 is exposed to acidic condition there (21).

Characteristic LOX-1 surface structure engaged in OxLDL binding – "basic spine structure"

In comparing the protein sequence among the CTLD containing proteins, the sequence that constructs the ligand binding surface shows significant variation (22-24). This part is called as long loop region (LLR). The significant sequence variety in the LLR suggests the importance of the part in the specific ligand recognition of each CTLD protein. The surface structures for two well characterized CTLD proteins, NKG2D and Ly49A, are compared to demonstrate the variable surface structures (Fig. 4). For both proteins, the ligand binding parts were identified by the X-ray structures in the complex with their ligands (25,26), the parts are marked on the surface structures (Fig. 4). The surface structure of LOX-1 is apparently different from those of NKG2D and Ly49A, as expected from their LLR sequences (Fig. 4). The LLR among the CTLD containing proteins, thus,

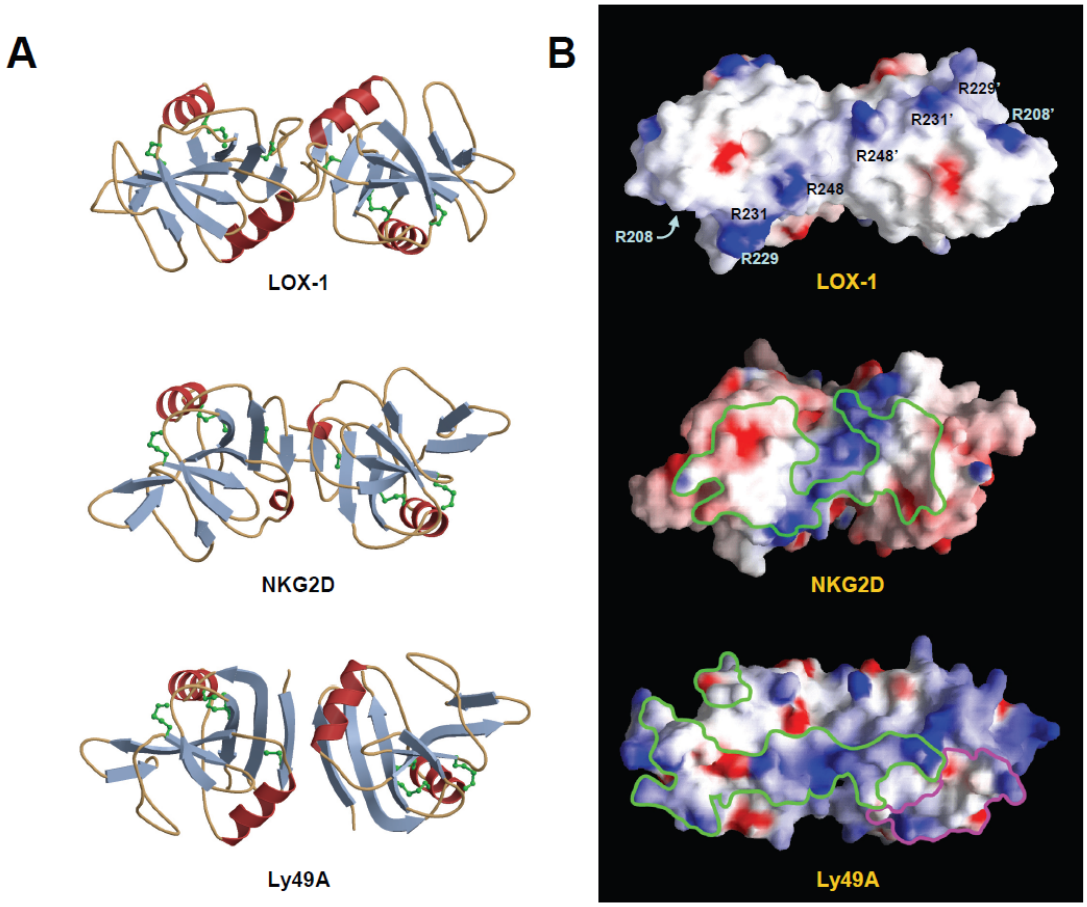

Fig. 4: Structure comparison of the LOX-1 dimer surface with other C-type lectin-like proteins; NKG2D (PDB: 1HYR) and Ly49A (PDB: 1QO3). (A) Ribbon representation of the CTLD dimer structure seen from the top of the dimer surface. (B) The surface charge distribution, the GRASP (34) representation, on the dimer surface with the corresponding view of the dimer structure shown in (A).

plays a homologous role to that of the hyper-variable loop of the immunoglobulin domain.

The detailed inspection into the structure of the disulfide-linked dimer of CTLD-NECK14 has shown that it has characteristically aligned Arg residues on the ligand binding surface which is mainly hydrophobic (Fig. 4). We call this remarkable arrangement of Arg residue on the surface as the “basic spine” structure (16). The native LDL particle is neutral, but when oxidized it becomes negatively charged. Therefore, it is functionally reasonable to think that the basic region on the LOX-1 ligand binding surface is engaged in the interaction with OxLDL. To explore the functional role of the basic spine structure of LOX-1, we achieved a series of OxLDL binding assays using LOX-1 mutants having amino acid replacement to the residues in the basic spine structure (16). The results have clearly shown that LOX-1 mutants, including R208N, R229N and R248N have shown the reduced ligand binding activities. The remarkable result was observed for R231N mutant, which has almost lost the binding ability. Together all of the results, we concluded that the residues in the basic spine structure are responsible for the OxLDL binding.

Among the mutant LOX-1s we analyzed, we found another interesting mutant, W150A, which has almost lost the biding activity to OxLDL (16). Although W150 resides beneath the binding surface, it is remarkable that it resulted in the almost complete activity loss. Careful inspection into the structure around the residue W150 has shown that there is a vacant cavity in the dimer interface (Fig. 5). As seen in the structure, W150

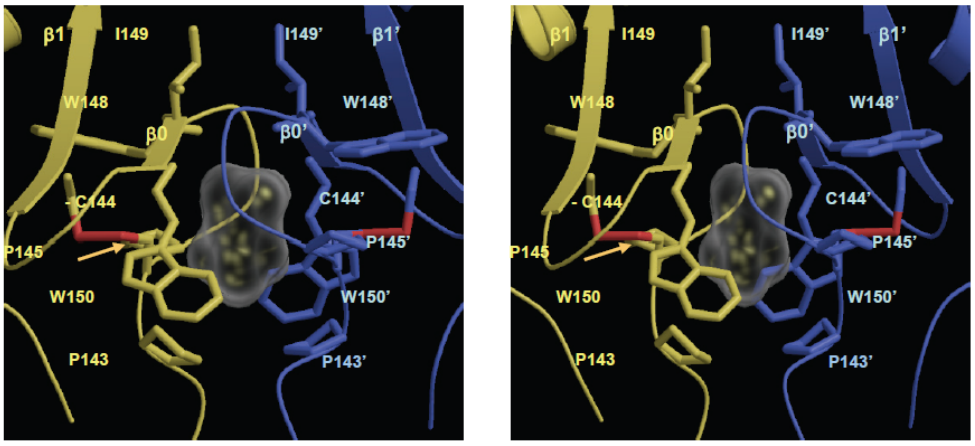

Fig. 5: The empty cavity found at the dimer interface of the LOX-1 CTLD-NECK14 structure, stereo view.

with other hydrophobic residues including P143, P145 and W148 form the cavity. In particular, W150 supports the bottom. The amino acid change from W to A may resize the cavity by reducing the side chain size. The resized cavity may accompany with domain disarrangement, which may disrupt the ligand-binding surface structure that harbors the basic spine, which may explain the observed activity loss (16).

Binding mode of LOX-1 to OxLDL

We have experimentally shown that the basic spine structure on the LOX-1 dimer surface is essential for OxLDL binding (16). The linear arrangement of basic residues inspired us to think over that LOX-1 may recognize the specific structural units on OxLDL particle.

LDL particle is a cholesterol carrier in blood flow, which consists of lipids and a single polypeptide chain comprising approximately 4,500 residues (27). The protein component in the LDL particle is apolipoprotein B-100 (apoB-100). No high resolution structural data are available for the LDL particle. Based on the amino acid sequence of apoB-100 and the image obtained from immunoelectron microscopy using apoB-100 specific monoclonal antibody, a model structure for LDL particle is proposed (Fig. 6) (27). The consensus model for the LDL particle shows that apoB-100 protein has a pentapartite domain

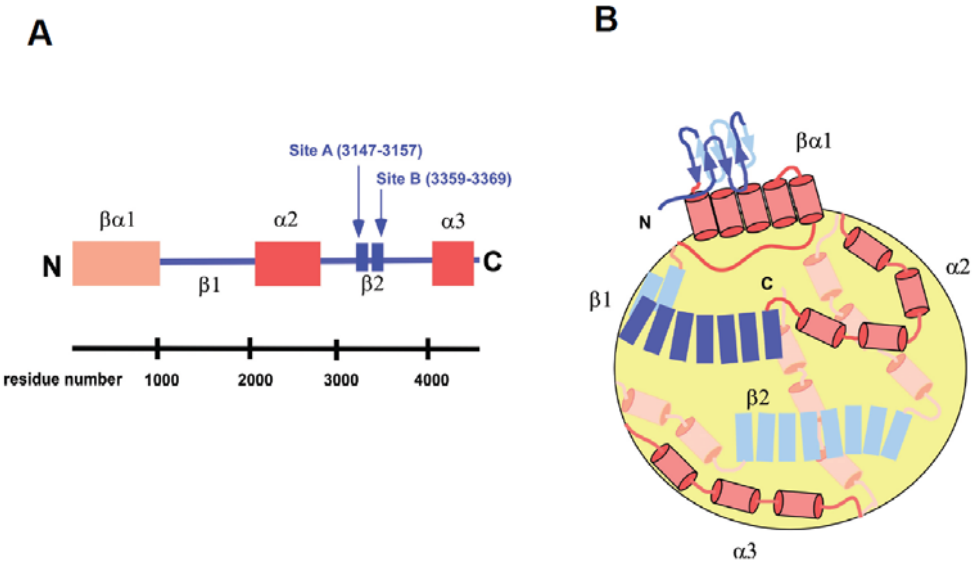

Fig. 6: Schematic presentation of an LDL particle. (A) The domain structure of apoB-100 predicted from its amino acid sequence (27). (B) The schematic drawing of the LDL particle model structure; amphipathic α-helices and β-sheets are spread over the LDL surface.

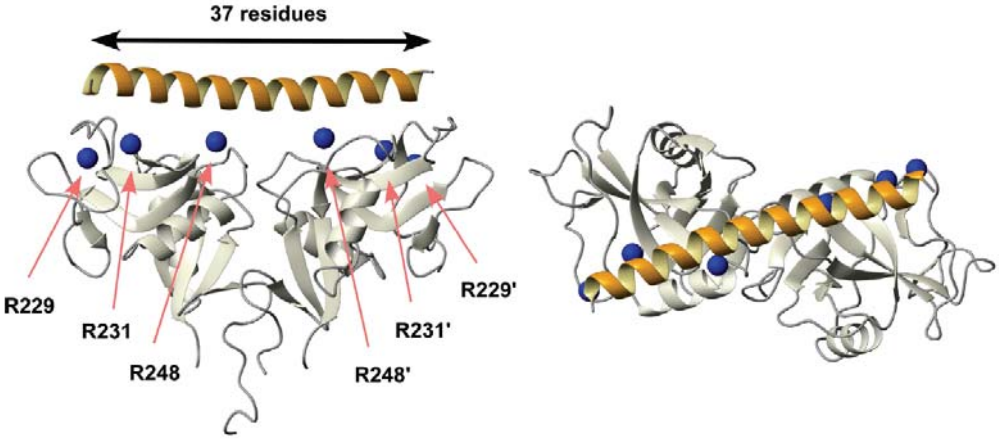

Fig. 7: LOX-1 dimer surface and an amphipathic α-helix interaction model. A 37-residue α-helical structure covers the entire basic spine structure on the LOX-1 ligand binding surface.

structure, NH_2-βα$_1$–β$_1$–α$_2$–β$_2$–α$_3$-COOH. The α and β domains are mainly composed of amphipathic α helices and β sheets. The amphipathic β1 and β2 domains display sequence characteristics typical for that of irreversible lipid association. On the other hand, α2 and α3 domains show similar sequence properties to that of an exchangeable apolipoprotein, such as apoA-I and apoE, and are assumed to be flexible domains with reversible lipid affinity (28). Estimate based on the surface area of LDL suggests the approximate number of amphipathic a helices is in the range 51 to 65. Thus, LDL particle posses a large number of amphipa thic α-helices on its surface. The schematic representation of the model structure for LDL particle is shown in Fig. 6.

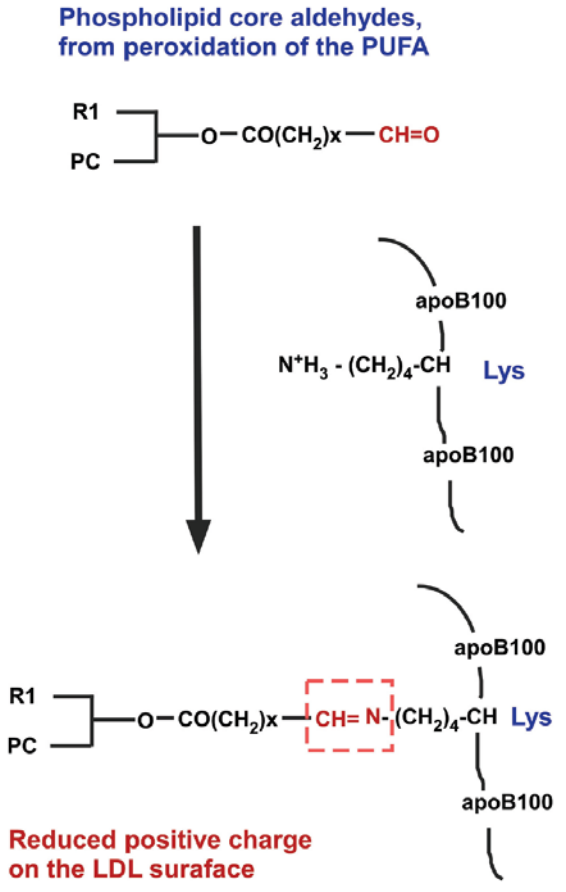

Fig. 8: Oxidation of polyunstaturated fatty acid and the generation of lipid-lysine conjugate at ε-amino group of lysines.

The basic spine structure of LOX-1, which exposes linearly arranged ARG residues, seems to be an appropriate platform to interact with an α-helix on the LDL. A 37 residue α-helix can fully cover the basic spine structure on the LOX-1 ligand binding surface (Fig. 7). Considering the complementary structural feature, it is reasonable to envisage that LOX-1 preferentially binds to an amphipathic α helix on the LDL particle.

During oxidation of LDL, there is a progressive decrease in the number of lysine ε-amino groups (29). The oxidation of polyunsaturated fatty acids can form aldehydes that subsequently modify the ε-amino groups of lysines to produce malondialdehyde and 4-hydroxynonenal. The blocking of lysine ε-amino groups through fatty acid oxidation, giving hydroxyl fatty acids, generates negatively charged LDL particles (Fig. 8). LOX-1 binds to AcLDL with comparable affinity, at least *in vitro*. Acetylation also blocks the lysine ε-amino groups without any side reactions. Oxidation and

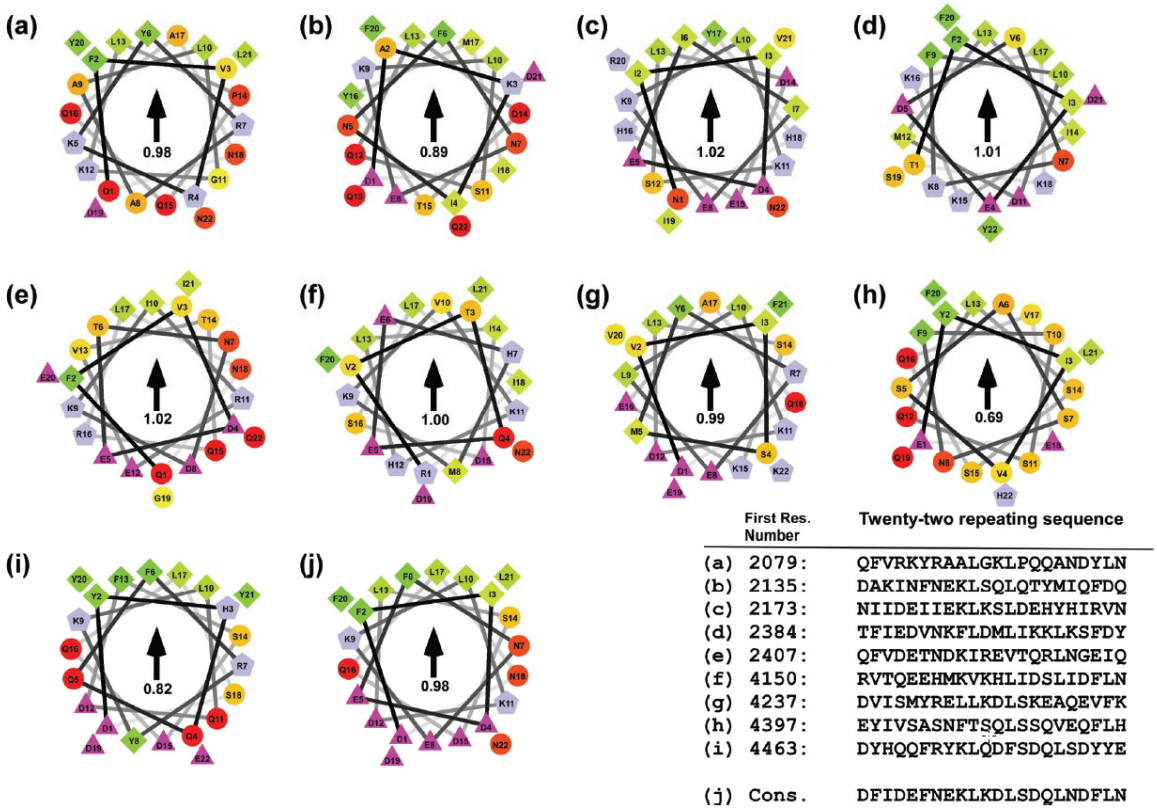

Fig. 9: Helical wheel presentation of the repeating 22-residue amphipathic sequences in apoB-100, which have been identified by computer analysis (28). The found repeating homologous 22-residue sequences with their starting residue number are listed in the table inserted in the figure.

acetylation, thus, produce the same effect with regard to the surface charge on the LDL particle. The fact that LOX-1 binds to both types of modified LDL with the same affinity *in vitro* suggests that the binding of LOX-1 to OxLDL is accelerated by the enhancement of the surface negative charge on the LDL. Amino acid composition of apoB-100 supports the idea that the modification of lysine residues reduces the surface positive charge. ApoB-100 comprises the following content of charged residues: Asp (5.1%), Glu (6.5%), Lys (7.8%) and Arg (3.3%), giving an almost neutral total surface charge in the native LDL. Taking into consideration the higher content of Lys over Arg, the blocking of the Lys ε-amino groups should chiefly affect the surface charge distribution on LDL.

Using computer analysis, De Loof and coworkers noted a 22-mer repeating sequence in the α2 and α3 amphipathic helical regions (28). It is evident that the repeating 22-mer amphipathic helical sequences display a common feature in having a negatively charged surface exposed to solvent (Fig. 9). This property is clearly demonstrated in the consensus sequence of these 22-mer repeating units. These structural features of apoB-100 complement those found at the interaction site on LOX-1, the basis spine structure. Thus, the 22-mer amphipathic helices, with negative surface charge, are likely to constitute the primary recognition sites with LOX-1.

Assembly of LOX-1 on the cell surface

Chemical cross linking experiments show that LOX-1 assembles as a hexamer on the cell surface, made up of three homodimeric LOX-1 (20). Our experiments using surface Plasmon resonance (SPR) have shown that the monomeric form of LOX-1, comprising a single CTLD lacking an inter-chain disulfide bond, does not show significant affinity (K_d = 1.5 x 10^{-2} M) for OxLDL fixed to a sensor chip via anit-apoB-100 antibody (Ohki et al., unpublished). However, the homodimeric form of LOX-1 with an inter-chain disulfide bond, as it does on the cell surface, shows significant affinity (K_d = 2.2 x 10^{-5}M) for OxLDL.

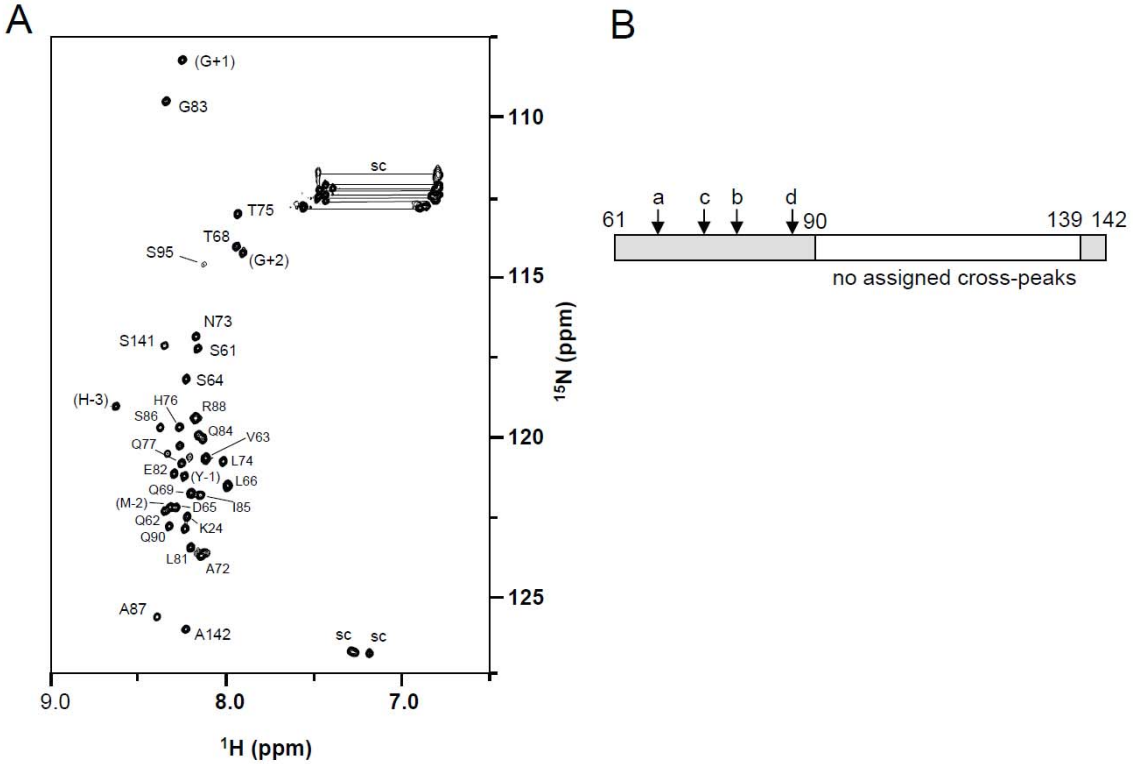

Fig. 10: Chimeric structure stabilities of LOX-1 NECK domain (33). (A) ^{1}H-^{15}N HSQC for the ^{15}N labeled NECK fragment, which exists as a disulfide-linked homodimer, shows only the signals coming from the N-terminal protease-sensitive part. (B) The parts in shadow give the clear NMR signals on the ^{1}H-^{15}N HSQC spectrum. The N-terminal shadowed region is protease-sensitive part; the arrows indicate the sites cleaved by proteases.

These results demonstrate that the homodimeric form of LOX-1 is essential for exerting the significant affinity for the ligand. This observation is consistent with the structure of the LOX-1 ligand binding surface made up of homodimeric form of the CTLDs. Our SPR experiments have shown the functional significance of the homodimeric form of LOX-1, but the observed affinity for the dimeric form of LOX-1 to OxLDL was far weak compared with those estimated for the LOX-1 on the cell endothelial cells, which was estimated to be a K_d = 1.7 x 10^{-8}M. This large difference in the observed binding activities between *in vitro* and *in vivo* should be ascribed to the assembly of LOX-1 on the cell surface. Indeed, in our SPR experiments using LOX-1 doped on the SPR sensor chip to achieve local high concentration of the protein, we could observe the apparent affinity of the condensed LOX-1 on the surface was improved to give a K_d = 7.5 x 10^{-10}M. This i*n vitro* observation infers that the local assembly of the LOX-1 is essential for the ligand binding activity to specifically interact with OxLDL.

The requirement for the receptor assembly found for LOX-1 is closely related to that found for the ligand recognition mode of DC-SIGN on dendritic cells (30,31). DC-SIGN, another member of the C-type lectin family of receptor proteins, has the same domain architecture as LOX-1, comprising NECK and CTLD domains for the extracellular part. DC-SIGN forms a tetramer at the NECK domain. Almost no binding to virus sized particles occurs when DC-SIGN is randomly distributed on the cell surface. However, organization of the protein into micro-domains, or lipid rafts, on the cell membrane allows biding of DC-SIGN to virus particles with significant affinity (32).

LOX-1 binding to OxLDL in a cluster infers that LOX-1 simultaneously binds to multiple sites on OxLDL. This is in the contrast to the case for the native LDL receptor where a single apoB-100 region, so called site B, is responsible for the receptor interaction; the native LDL receptor interacts with LDL particle as a single molecule. In

considering the requirement for the multiple-site biding of LOX-1 to OxLDL particle, our proposing LOX-1 binding mode to OxLDL is supported, where clustered LOX-1 receptors cooperatively bind to negatively charged amphipathic α-helices on OxLDL.

Chimerical structural stabilities in the NECK domain

The NECK domain in LOX-1 shows sequence similarity to the coiled-coil region of myosin. Because of this sequence similarity, the NECK region has been assumed to form a rod-like structure as found for the corresponding part of myosin. We have extensively analyzed the structural features of the NECK domain of LOX-1 using various methods including limited proteolysis, chemical cross-linking, circular dichroism (CD) and NMR (33). Our results obtained have revealed the unique structural characters of the LOX-1 NECK domain, which was not unexpected from its sequence.

We have shown the LOX-1 NECK does not have a uniform rod-like structure (33). Although not random, one-third of the N-terminal NECK is less structured than the remainder of the domain, which part has shown the high sensitivity to protease digestion. The remaining C-terminal part of the NECK shows a coiled-coil structure, but the coiled-coil dimer form is not stable but in dynamic equilibrium among multiple conformational states on a μs – ms time scale (33). The structure dynamics was revealed by NMR (Fig. 10). This chimerical structural stability found for the LOX-1 NECK should facilitate the LOX-1 ligand recognition to OxLDL, which is clustered on the cell surface (Fig. 11).

Conclusion

We have described the first structure of the OxLDL receptor of the C-type lectin like family of proteins. The characteristic basic spine structure found on the dimer surface of LOX-1 has shown to be responsible for OxLDL binding. Based on the consensus model structure of LDL particle, we have proposed the LOX-1 binding mode to OxLDL. In our proposal, the basic spine structure in LOX-1 may interact with negatively charged amphipathic α-helices which are repeated in apoB-100 that wraps the lipid particle to form LDL particle. Oxidation to LDL produces a lysine-lipid conjugate, which concomitantly reduces the surface positive charge. The lipid-lysine conjugate formation, thus, facilitates the LOX-1 – OxLDL interaction through negatively charged amphipathic α-helices on OxLDL particle. *In vivo*

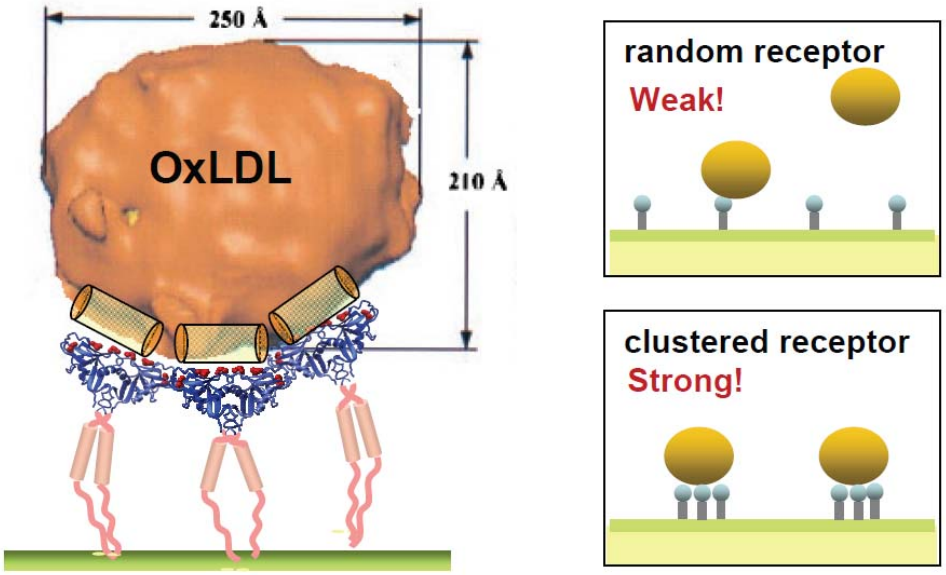

Fig. 11: Schematic drawing of LOX-1 binding to OxLDL on the cell surface. Clustered LOX-1 on the cell surface can bind to negatively charged amphipathic α-helices on OxLDL with significant affinity by using the structural flexure in the NECK domain.

and *in vitro* experiments have shown that LOX-1 clusters on the cell surface and the clustering of LOX-1 seems essential for its specific binding to OxLDL. The clustered LOX-1 on the cell surface should bind to the multiple sites on the OxLDL particle, where the repeating negatively charged amphipathic α-helices should be engaged in the interaction. In achieving the multiple-site binding to OxLDL by LOX-1 clustered on the cell surface, LOX-1 should require structural flexibility for efficient grasping the amphipathic helices on the LDL particle. Our structural analyses on the LOX-1 NECK domain have shown that the NECK has an appropriate structural flexure to accelerate the LOX-1 binding to OxLDL. The N-terminal part of the NECK is more flexible than the remaining C-terminal coiled-coil part, although the latter part is not stable but in dynamic equilibrium among multiple conformations. Our proposing binding mode of LOX-1 to OxLDL is schematically depicted in Fig. 11. Because LOX-1 is one of the critical receptor molecules in atherogenesis, the continuing efforts in understanding the interaction between LOX-1 and OxLDL will make clearer view on the LOX-1 ligand recognition mode and provide advantages in developing therapeutic approached for the atherosclerosis.

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