Ligand Specificity of LOX-1, a Novel Endothelial Receptor for Oxidized Low Density Lipoprotein

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Abstract—Endothelial dysfunction, or activation, elicited by oxidized low density lipoprotein (Ox-LDL) and its lipid constituents has been shown to play a key role in the pathogenesis of atherosclerosis. We recently have identified a novel receptor for Ox-LDL-designated lectin-like Ox-LDL receptor (LOX-1) in vascular endothelial cells. To examine ligand specificity of LOX-1, we established CHO cell lines stably expressing both human and bovine LOX-1 (LOX-1-CHO). LOX-1-CHO bound and degraded 125I-labeled Ox-LDL but did not significantly degrade 125I-labeled acetylated LDL (Ac-LDL). Fucoidin and maleylated BSA (M-BSA), which inhibit 125I-Ox-LDL binding to class A scavenger receptors, did not inhibit 125I-Ox-LDL binding or degradation in LOX-1-CHO. Polyinosinic acid and carrageenan, in contrast, significantly reduced 125I-Ox-LDL binding to LOX-1-CHO by 62% and 60%, respectively. Delipidated and untreated 125I-Ox-LDL were bound and degraded equally in LOX-1-CHO; furthermore, excess amounts of unlabeled, delipidated Ox-LDL inhibited binding and degradation of untreated 125I-Ox-LDL. Taken together, LOX-1 is a receptor for Ox-LDL but not for Ac-LDL. LOX-1 recognizes protein moiety of Ox-LDL, and its ligand specificity is distinct from other receptors for Ox-LDL, including class A and B scavenger receptors. (Arterioscler Thromb Vasc Biol. 1998;18:1541-1547.)

Key Words: endothelial cell ■ LOX-1 ■ oxidized LDL ■ atherosclerosis

Several lines of evidence have suggested that oxidized low density lipoprotein (Ox-LDL) plays a pivotal role in the pathogenesis of atherosclerosis. Ox-LDL and its lipid constituents appear to modulate endothelial functions that are relevant to atherogenesis. Vascular endothelial cells in culture and liver sinusoidal endothelial cells have been shown to take up modified forms of LDL, including Ox-LDL. Although several different molecules, such as class A macrophage scavenger receptors, CD36, CD68, SR-BI, MARCO, and macrosialin (CD68), have been identified to support Ox-LDL uptake in macrophages; molecular mechanisms involved in endothelial uptake of Ox-LDL remain to be fully clarified. Using expression cloning strategy, we recently have identified a novel molecule, designated lectin-like Ox-LDL receptor-1 (LOX-1) in cultured bovine aortic endothelial cells. LOX-1 is a 50-kDa type II membrane glycoprotein whose structure belongs to the C-type lectin family and does not share any structural homology with other known molecules that can act as a receptor for Ox-LDL. LOX-1 also is expressed in arterial endothelial cells in humans in vivo, suggesting that LOX-1 may play an important role in the pathogenesis of atherogenesis and vascular diseases.

Scavenger receptors, in general, have been shown to exhibit broad ligand specificity. They can recognize modified forms of LDL, such as Ox-LDL, Ac-LDL, and maleylated LDL, as well as 4-stranded nucleic acids (polyinosinic acid), polysaccharides (dextran sulfate, fucoidin), and phospholipids (phosphatidylserine). Recent reports also have shown that some of the scavenger receptors can take up advanced glycosylation end products, oxidatively damaged erythrocytes, apoptotic cells, lipopolysaccharide, and beta-amyloid. In this report, we show ligand specificity of this novel Ox-LDL receptor, LOX-1, by use of Chinese hamster ovary K1 (CHO-K1) cells stably expressing bovine and human LOX-1.

Methods

Materials
Polyinosinic acid (poly I), polycytidylic acid (poly C), fucoidin, carrageenan (type III Kappa), chondroitin sulfate A, and n-octyl-β-D-glucopyranoside (octyl glucoside) were purchased from Sigma. Lipoperoxide test kits for thiobarbituric acid reactive substances (TBARS) were obtained from Wako Pure Chemical. Agarose gel plates and running buffer for electrophoretic mobility of lipoproteins were purchased from Helena laboratories. Reagents for enzymatic determination of total cholesterol were purchased from Toyobo.

Lipoprotein and Ligand Preparations
Human LDL (1.019 to 1.063 g/mL) was isolated from the plasma of healthy human subjects by sequential ultracentrifugation at 4°C. LDL was dialyzed against 3 changes of LDL buffer (150 mmol/L NaCl; 0.24 mmol/L EDTA; pH 7.4) for 36 hours at 4°C, filtered

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through 0.45-μm filters, and stored at 4°C. After extensive dialysis against 3 changes of PBS for 36 hours at 4°C, oxidative modification of LDL was performed by incubating with 7.5 μmol/L CuSO₄ at 37°C for 24 hours. Acetylation of LDL was achieved by repeated additions of acetic anhydride as previously described. Ox-LDL and acetylated LDL (Ac-LDL) were dialyzed against 3 changes of LDL buffer for 36 hours at 4°C. Modification of LDL was monitored by electrophoretic mobility relative to native LDL expressed as relative electrophoretic mobility (REM; arbitrary unit). REM for Ox-LDL and Ac-LDL were 2.20 ± 0.11 (n = 5) and 2.23 ± 0.21 (n = 5), respectively. Oxidation of LDL was further monitored by the amounts of lipid peroxides by measuring TBARS. Values for TBARS in Ox-LDL, Ac-LDL, and native LDL were 9.76 ± 1.34 nmol (n = 6), 1.68 ± 0.60 nmol (n = 2), and 1.15 ± 0.21 nmol (n = 2) of MDA/mg protein, respectively. Ox-LDL and Ac-LDL were radiolabeled with Na¹²⁵I (DuPont) by the iodine monochloride method previously described. Maleylated BSA (M-BSA) was prepared as described previously. Protein concentrations were determined by the method of Lowry et al.

Cell Culture and Stable Transfection
CHO-K1 cells were cultured in Ham’s F12 medium supplemented with 10% (v/v) fetal bovine serum (FBS, Irvine Scientific), 100 U/mL penicillin, and 100 μg/mL streptomycin (medium A) at 37°C in a humidified air with 5% CO₂. A bovine LOX-1 (BLOX-1) expression vector, designated pBLOX-1 (1 μg) was cotransfected with pSV2bssr (10 ng) (Funakoshi) into CHO-K1 cells by calcium phosphate transfection method. Colonies resistant to blasticidin S in medium B (medium A containing 10 μg/mL of blasticidin S) were screened by intracellular uptake of fluorescent DiI-labeled Ox-LDL (5 μg/mL for 2 hours in medium B) to identify BLOX-1-positive cells. One of these positive cells was picked and used as stable transfectant of BLOX-1 (BLOX-1-CHO). A CHO-K1 cell line stably expressing human LOX-1, which is a human homolog of BLOX-1, was also established (HLOX-1-CHO). A CHO-K1 cell line stably expressing murine class A scavenger receptor type II (mSR-II-CHO) was kindly provided by Dr Tatsuhiko Kodama (University of Tokyo, Japan).

Binding and Degradation of ¹²⁵I-Labeled Lipoproteins
Binding of ¹²⁵I-Ox-LDL and ¹²⁵I-Ac-LDL to BLOX-1-CHO, HLOX-1-CHO, mSR-II-CHO, and nontransfected CHO-K1 (control CHO) was measured after incubation in 12-well culture dishes at 4°C as previously described. In brief, after washing 3 times with PBS, adherent cells were prechilled in 1 mL ice-cold DMEM supplemented with 10 mmol/L HEPES-NaOH (pH 7.4) and 10% (v/v) FBS (medium C) for 30 minutes at 4°C. Culture media were then removed and replaced with 0.5 mL of medium C containing the indicated amounts of ligands. After incubation at 4°C for 2 hours, the conditioned media were removed, and the cells were washed rapidly 3 times with Tris washing buffer (50 mmol/L Tris-HCl; 150 mmol/L NaCl; pH 7.4) containing 2 mg/mL of BSA, followed by 2 washes for 10 minutes and 2 rapid washes with Tris washing buffer without BSA. The cells were then dissolved in 0.5 mL of 0.2 N NaOH for 3 to 4 hours on a shaker, and the radioactivity associated with cells was measured by a gamma counter. Proteolytic degradation of ¹²⁵I-labeled lipoproteins was carried out as previously described. In brief, cells were incubated with ligands in medium C at 37°C for 4 to 6 hours, and radioactivities in trichloroacetic acid (TCA)-soluble, chloroform-unextractable fractions in the cell-conditioned media were measured. Each value obtained from medium incubated with lipoproteins without cells was subtracted from that obtained from cell-conditioned medium incubated with lipoproteins.

Delipidation of Lipoproteins
Ox-LDL and ¹²⁵I-Ox-LDL were delipidated by the method of Bligh and Dyer as described by Parthasarathy et al. The apolipoprotein was solubilized in an aqueous solution using octyl glucoside (6.0

Figure 1. Dose-response relationship in ¹²⁵I-Ox-LDL binding to BLOX-1-CHO. BLOX-1-CHO and control CHO were incubated in duplicate with indicated concentrations of ¹²⁵I-Ox-LDL for 2 hours at 4°C in the presence or absence of a 100-fold excess amount of unlabeled Ox-LDL. The specific binding was calculated by subtracting the values in the presence of an excess amount of unlabeled Ox-LDL from the values in the absence of unlabeled Ox-LDL. Data are representative of 3 separate experiments. For Scatchard analysis, values of specific binding to control CHO were subtracted from values of specific binding to BLOX-1-CHO to obtain the specific binding activities mediated by BLOX-1.
mg/mL, 30 times more than protein concentration). Any turbidity usually was cleared by the addition of NaOH. The solution was dialyzed immediately against PBS for 24 hours at 4°C. To confirm that delipidation was completed, cholesterol content of delipidated Ox-LDL (Ox-apoB) and untreated Ox-LDL was measured by an enzymatic assay. Cholesterol was undetectable in delipidated Ox-LDL samples.

Results

LOX-1 Binds and Degrades Ox-LDL but Not Ac-LDL

Dose-response relationship in $^{125}$I-Ox-LDL binding to BLOX-1-CHO showed saturation kinetics (Figure 1), and Scatchard analysis revealed that $^{125}$I-Ox-LDL can bind to bovine LOX-1 with an approximate dissociation constant (Kd) of 36 μg/mL (Figure 1, inset). This result indicates that LOX-1 can bind Ox-LDL with relatively lower affinity than that (Kd < 10 μg/mL) in Ox-LDL binding to class A scavenger receptors. To examine whether LOX-1 also can recognize Ac-LDL, binding and proteolytic degradation of $^{125}$I-labeled lipoproteins in BLOX-1-CHO were examined. As shown in Figure 2A, BLOX-1-CHO showed prominent binding of $^{125}$I-labeled Ox-LDL as compared with control CHO. In contrast, binding of $^{125}$I-Ac-LDL to BLOX-1-CHO was not significantly different from that in control CHO (Figure 2A). Proteolytic degradation of $^{125}$I-Ox-LDL in BLOX-1-CHO showed similar results with those observed in binding studies (Figure 2B). Amounts of $^{125}$I-Ac-LDL degraded in BLOX-1-CHO were not significantly different from those in control CHO (Figure 2B). Furthermore, as previously reported, both binding and degradation of $^{125}$I-Ox-LDL were significantly displaced by a 50-fold excess amounts of Ox-LDL and Ox-LDL in mSR-II-CHO (Figure 3B). Addition of 50-fold excess amounts of unlabeled Ox-LDL abolished binding and degradation of $^{125}$I-Ox-LDL to BLOX-1-CHO by 74% and 86%, respectively. However, the same concentration of unlabeled Ac-LDL or native LDL did not block the binding and degradation of $^{125}$I-Ox-LDL to BLOX-1-CHO (Figure 3A). These results demonstrate that LOX-1 is a receptor for Ox-LDL but not for Ac-LDL.

Effects of Pharmacological Competitors on Ox-LDL Binding to LOX-1

To characterize ligand specificity of LOX-1, effects of various pharmacological competitors, which have been shown to inhibit Ox-LDL binding to class A and B scavenger receptors, on $^{125}$I-Ox-LDL binding to BLOX-1-CHO were examined. As previously reported, optimal concentrations of polyanions, such as poly I, carrageenan, fucoidin, and M-BSA, inhibited $^{125}$I-Ox-LDL binding to mSR-II-CHO by ~90% (Figure 4A). In contrast, no inhibitory effect of fucoidin or M-BSA on $^{125}$I-Ox-LDL binding to BLOX-1-CHO was observed (5% and 8% inhibition, respectively; Figure 4A). Poly I and carrageenan abolished $^{125}$I-Ox-LDL binding to BLOX-1-CHO by 62% and 60%, respectively, which was comparable with the inhibitory effect of excess amounts of unlabeled Ox-LDL (74% inhibition; Figure 4A). Competition studies in proteolytic degradation assays gave similar results with those found in binding assays (Figure 4B). Although poly I, carrageenan, fucoidin, and M-BSA significantly inhibited the degradation of $^{125}$I-Ox-LDL in mSR-II-CHO, fucoidin and M-BSA had no inhibitory effect on $^{125}$I-Ox-LDL degradation in BLOX-1-CHO. In contrast, poly I and carrageenan inhibited degradation of $^{125}$I-Ox-LDL by 70% and 50%, respectively in BLOX-1-CHO (Figure 4B).
These differences in the inhibitory effect of pharmacological competitors in Ox-LDL binding to LOX-1 suggest that molecular mechanisms of Ox-LDL binding to LOX-1 may be different from those binding to class A scavenger receptors.
LOX-1 Binds to Protein Moiety of Ox-LDL

To define an epitope(s) on Ox-LDL particles recognized by LOX-1, binding and degradation of delipidated Ox-LDL (Ox-apo B) in BLOX-1-CHO, as well as in HLOX-1-CHO, were examined. BLOX-1-CHO was able to bind delipidated 125I-Ox-apoB almost equally to untreated 125I-Ox-LDL (Figure 5A). Furthermore, binding of both 125I-Ox-LDL and 125I-Ox-apoB to BLOX-1-CHO was equally inhibited by a 50-fold excess amount of unlabeled Ox-LDL (by 66% and 42%, respectively) as well as unlabeled Ox-apoB (by 75% and 66%, respectively; Figure 5A). Similar results were obtained in binding and degradation of 125I-Ox-apo B and 125I-Ox-LDL in HLOX-1-CHO (Figure 5B and 5C). These results indicate that LOX-1 recognizes protein moiety of Ox-LDL and that lipid constituents of Ox-LDL do not appear to be necessary for Ox-LDL binding to LOX-1.

Discussion

Cellular uptake and subsequent proteolytic degradation of Ox-LDL in vascular endothelial cells, as well as macrophages and activated smooth muscle cells, appear to play a key role in atherogenesis. Previous studies have identified several classes of receptors for Ox-LDL that exhibit a broad spectrum of ligand specificities. The present study, therefore, sought to define ligand specificities of LOX-1, because this novel receptor for Ox-LDL does not share any structural homology with other known receptors for Ox-LDL.
Progression of oxidative modification of LDL is followed by generation of lipid peroxides. During lipid peroxidation, reactive aldehydes form Schiff bases with lysine residues on apolipoprotein B-100 and thereby increase the negative charge of LDL particles. Thus, negative charges on LDL particles produced during oxidative modification appear to facilitate binding to the positively charged collagen-like domain of class A scavenger receptors. Furthermore, class A scavenger receptors can recognize a wide spectrum of negatively charged macromolecules including Ox-LDL and Ac-LDL. The present data with CHO-K1 cells that stably express LOX-1, however, show that LOX-1 can bind and degrade comparable amounts of Ox-LDL but not significant amounts of Ac-LDL. Moreover, binding and degradation of Ox-LDL in these cells can be blocked effectively by poly I and carrageenan but not by fucoidin or M-BSA. Taken together, interactions between Ox-LDL and LOX-1 cannot be explained simply by negative charges of Ox-LDL particles but rather depend on other molecular mechanisms.

Class A scavenger receptors recognize epitopes on apoprotein B-100 of modified LDL, CD36, in contrast, does not bind delipidated Ox-LDL, indicating that CD36 recognizes the lipid portion of Ox-LDL but not oxidized apolipoprotein B-100. As shown in this study, LOX-1 appears to bind to the protein portion of Ox-LDL. Our studies cannot exclude completely the possibility that LOX-1 may recognize certain lipids that are firmly linked to apolipoprotein B-100 and therefore was not able to be extracted by organic solvents; however, these results clearly indicate that molecular mechanisms of Ox-LDL binding to LOX-1 are distinct from those of class B scavenger receptors, such as CD36 and SR-BI.

Because LOX-1 has a lectin-like structure in the extracellular domain, like selectins, certain sugar chains on oxidatively modified apolipoprotein B-100 might be responsible for binding to LOX-1. Our preliminary experiments, however, failed to show any significant inhibition in Ox-LDL binding to BLOX-1-CHO by oligosaccharides that have been shown to bind to selectins (data not shown). Further studies are necessary to elucidate molecular mechanisms involved in binding and internalization of Ox-LDL by LOX-1, which has a simple C-type lectin-like structure. Moreover, scavenger receptors, in general, have been shown to bind a variety of pathophysiological ligands, such as oxidized RBC, apoptotic cells, advanced glycosylation end products, lipopolysaccharide, and beta-amyloid. Studies in progress in our laboratory will determine whether LOX-1 can act as a receptor for these ligands.

In summary, this article demonstrates the unique ligand specificity of LOX-1, a novel endothelial receptor for Ox-LDL. Elucidation of molecular mechanisms involved in receptor-ligand interactions of LOX-1 may provide a new therapeutic target in atherogenesis and vascular diseases.

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