Expression of Lectinlike Oxidized Low-Density Lipoprotein Receptor-1 in Human Atherosclerotic Lesions

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Background—Oxidized LDL (Ox-LDL) seems to play key roles in atherogenesis. Lectinlike Ox-LDL receptor-1 (LOX-1) is a recently identified cell-surface receptor for Ox-LDL. The relationship of this novel receptor for Ox-LDL to atherogenesis, however, has not yet been clarified. In this study, we explored the expression of LOX-1 in the atherosclerotic lesions of human carotid arteries.

Methods and Results—Using carotid endarterectomy specimens obtained from 21 patients and 2 samples of normal human aortas, we examined LOX-1 expression by reverse transcription–polymerase chain reaction and immunohistochemistry. In aortas without atherosclerosis, LOX-1 expression was undetectable by immunohistochemistry and negligible by reverse transcription–polymerase chain reaction. In carotid arteries, luminal endothelial cells covering early atherosclerotic lesions were more frequently positive for LOX-1 expression than those in advanced atherosclerotic lesions. Endothelial cells in the intimal neovascularization of advanced lesions also expressed LOX-1. In addition, macrophages and smooth muscle cells in the intima of advanced atherosclerotic plaques were positive for LOX-1 expression.

Conclusions—LOX-1 may play important roles in Ox-LDL uptake and subsequent functional alteration in the luminal endothelium in early atherosclerotic lesions and in intimal neovascular endothelial cells in advanced plaques. Furthermore, LOX-1 may also be involved in Ox-LDL uptake and subsequent foam cell transformation in macrophages and smooth muscle cells in the atherosclerotic intima. (Circulation. 1999;99:●●●●)

Key Words: atherosclerosis ■ cholesterol, LDL ■ immunohistochemistry

Several lines of evidence suggest that oxidized LDL (Ox-LDL) may play crucial roles in the pathogenesis of atherosclerosis.1–5 The cellular uptake of Ox-LDL by macrophages and activated smooth muscle cells seems to transform these cells into foam cells, which accumulate in the atherosclerotic intima. In addition, the endothelial cell activation elicited by Ox-LDL and its lipid constituents has been implicated in atherogenesis. Class A macrophage-scavenger receptors (MSR-A) were the first to be identified as receptors for the cellular uptake of acetylated LDL and Ox-LDL.6,7 Subsequent studies have investigated CD36,8,9 scavenger receptor class B type 1 cells,10,11 and macroscalin/CD68.12–14 In vascular endothelial cells, previous studies have suggested that the endothelial uptake of Ox-LDL seems to depend on cell-surface receptors, which may be encoded by different genes from known scavenger receptors.15,16 We recently identified a novel receptor for Ox-LDL, designated C-type lectinlike Ox-LDL receptor-1 (LOX-1), in cultured bovine aortic endothelial cells.17 Human17 and murine18 homologues of LOX-1 have been cloned and sequenced by screening human and murine lung cDNA libraries.

LOX-1 is a type II membrane protein that belongs to the C-type lectin family.17 LOX-1 recognizes Ox-LDL, polyinosinic acid, and carrageenan, but not acetylated LDL, fucoidin, or maleylated BSA.19 The expression of LOX-1 can be induced by the inflammatory cytokine tumor necrosis factor-α and phorbol ester.20 In addition, fluid shear stress also induces LOX-1 expression in cultured vascular endothelial cells.21 These data indicate that LOX-1 expression is dynamically regulated by pathophysiological stimuli relevant to atherogenesis and inflammation.

Previous studies have shown that MSR-A is expressed by macrophages in atherosclerotic lesions.22–24 In the vascular endothelium in various stages of atherogenesis, molecular markers of endothelial activation, including intercellular adhesion molecule-1,25,26 vascular cell adhesion molecule-1,27,28 E-selectin,29 and P-selectin,30 are expressed in human atherosclerotic lesions.

In the present study, therefore, we explored the expression of LOX-1 in the atherosclerotic lesions of human carotid arteries. We provide evidence that LOX-1 is
expressed by luminal endothelial cells, neovascular endothelial cells, and nonendothelial cells in human atherosclerotic lesions.

Methods

Tissue Sampling
Fresh-frozen sections were prepared from human carotid endarterectomy specimens from 21 patients (18 men and 3 women between 51 and 74 years of age) who had transient ischemic attacks or minor completed strokes before their operations. These endarterectomy specimens contained both early atherosclerotic lesions distant from the center of the plaque and advanced lesions consisting of fibrofatty plaques; therefore, these specimens were carefully excised to avoid disruption of the fragile endothelium covering the luminal surface. They were then divided into 2 pieces so that each piece contained either an early lesion with some endothelial infiltrates or an advanced atheromatous lesion. Fragments of human aortas without visible atherosclerotic lesions were obtained from 2 patients (a 60-year-old man and a 67-year-old woman) who underwent cardiovascular surgery. Tissue samples were immediately snap-frozen in isopentane prechilled with dry ice, embedded in OCT compound (Sakura Finetech), and stored at −80°C until use. RNA was isolated from portions of 8 carotid endarterectomy samples and 2 aortic tissue samples. These 8 carotid endarterectomy samples contained both early lesions and advanced lesions.

Reverse Transcription–Polymerase Chain Reaction Analysis
Total cellular RNA was isolated from 8 endarterectomy samples and 2 aortic tissue samples using the method of Chomczynski and Sacchi29 after homogenization with Trizol reagent (Gibco). Total cellular RNA (250 ng) was reverse-transcribed with random hexamer using SuperScript (Gibco). The transcribed cDNA was used as polymerase chain reaction (PCR) amplification with specific primers for human LOX-1 (hLOX-1) and β-actin. Two specific primers corresponding to the published sequences17,30 were used to amplify both hLOX-1 (5′-TACCTCTCATGTGGTGTC C-3′ and 5′-AGTTCTGCAGC CAGCTAATGACAG-3′) and β-actin (5′-TGACGGGCTAC CCAAACGTG G GCCCATCTA-3′ and 5′-CTAGAAGCATTGC GTGGGA CGATGG-3′). PCR amplification was performed by 35 cycles of denaturation, annealing, and elongation with Taq DNA polymerase (New England Biolabs, Inc). PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

Generation of Anti-LOX-1 Monoclonal Antibodies
A cDNA fragment corresponding to the extracellular domain of hLOX-1 (amino acid numbers 85 through 273) was amplified by PCR and subcloned into pQE 32 vector (Qiagen). The 6xHis-tagged recombinant protein was expressed in Escherichia coli, purified with Ni-NTA resin (Qiagen), and used as an antigen to immunize mice. Hybridomas were prepared by standard procedures and screened by ELISA and immunoblot.

Anti-bovine LOX-1 (bLOX-1) monoclonal antibody, which cross-reacts with hLOX-1, was used for double-labeled immunohistochemistry. This antibody was generated by immunizing rats with Chinese hamster ovary (CHO) cells stably expressing bLOX-1.17 Hybridomas were screened by cell-surface immunobinding to CHO cells stably expressing bLOX-1. Cross-reactivity to hLOX-1 was expressed by luminal endothelial cells, neovascular endothelial cells, and nonendothelial cells in human atherosclerotic lesions.

Figure 1. RT-PCR detection of LOX-1 mRNA in human atherosclerotic plaques. The total RNA (250 ng) from 8 atherosclerotic plaques and 2 aortas was first reverse-transcribed. cDNA fragments were amplified by 35-cycle PCR with set of primers for hLOX-1 (top) and β-actin (bottom). PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis. Lane 1, DNA size marker; lanes 2 and 3, normal aorta; lanes 4 through 11, atherosclerotic plaques.

Transient Transfection of hLOX-1 cDNA into CHO Cells
One milligram of the mammalian expression plasmid containing the full length of hLOX-1 cDNA17 was transfected into CHO cells cultured in Laboratory-Tek chamber slides (Nalge Nunc International) by a lipofection method using Lipofectamine Plus (Gibco). Cells were incubated for 48 hours after transfection; fixed with cold acetone; washed with PBS; and incubated with anti-hLOX-1 monoclonal antibody, anti-bLOX-1 monoclonal antibody, nonimmune mouse IgG (Zymed), or nonimmune rat IgG (Zymed) (1:100 dilution) for 1 hour, and then incubated with fluorescein isothiocyanate–conjugated goat anti-mouse or anti-rat IgG antibody (Caltag Laboratories). After being mounted with PBS and 90% (vol/vol) glycerol, 0.1% (wt/vol) p-phenylenediamine, and 10 mmol/L NaCl, samples were covered with glass slips and subjected to fluorescence microscopy. Untransfected CHO cells were immunostained with the same antibodies to serve as controls.

Single-Labeled Immunohistochemistry
An avidin-biotin complex (ABC) immunoperoxidase technique was used as previously described.31 In brief, after being fixed with cold acetone, frozen sections were incubated with 0.1% BSA-PBS containing 2% normal horse serum and then with anti-hLOX-1 monoclonal antibody. The sections were then incubated with biotinylated secondary antibodies, Endogenous peroxidase activity was blocked by methanol containing 0.3% hydrogen peroxide, after which avidin-biotin peroxidase complexes (ABC Elite Kit, Vector Labs) were added. Antibody binding was visualized with 3,3′-diaminobenzidine tetrahydrochloride (Vector Labs) and then counterstained with Mayer’s hematoxylin. Cells immunostained with nonimmune mouse IgG (Zymed) served as negative controls.

Double-Labeled Immunohistochemistry
For double immunostaining, sections were first incubated with the anti-bLOX-1 monoclonal antibody and biotinylated anti-rat IgG, which was followed by incubation with an avidin-biotin peroxidase conjugate and 3,3′-diaminobenzidine tetrahydrochloride with nickel chloride (Vector Labs). Sections were subsequently incubated with primary antibodies for cell-type characterization, which was followed by incubation with alkaline phosphatase–labeled anti-mouse IgG and fast red alkaline phosphatase substrate solution (Vector Labs).

Results

Upregulated Expression of LOX-1 mRNA in Human Atherosclerotic Plaques
To examine the levels of LOX-1 mRNA expression in atherosclerotic plaques, total cellular RNA was isolated from

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an endarterectomy tissue specimen and subjected to reverse transcription–PCR (RT-PCR) analysis. As illustrated in Figure 1, LOX-1 mRNA was expressed in atherosclerotic plaques. In the unaffected aortas of humans, in contrast, negligible amounts of LOX-1 mRNA were detectable. Amounts of β-actin mRNA, which was used as an internal control, were not significantly different. These results thus indicate that LOX-1 mRNA expression is upregulated in human atherosclerotic lesions.

**Immunoreactivities of 2 Monoclonal Antibodies With hLOX-1**

A monoclonal antibody directed to hLOX-1 was generated by immunizing mice with recombinant protein containing the extracellular domain of hLOX-1. This anti-hLOX-1 monoclonal antibody can bind to hLOX-1 expressed on the cell surface of CHO cells transfected by hLOX-1 cDNA (Figure 2A). This monoclonal antibody did not react to untransfected CHO cells (Figure 2B). CHO cells expressing hLOX-1 were not stained with nonimmune mouse IgG (Figure 2C). The cross-reactivity of the anti-bLOX-1 monoclonal antibody to hLOX-1 was confirmed in the same way by immunofluorescence microscopy (Figure 2D through 2F).

**LOX-1 Expression in Luminal Endothelial Cells Was More Prominent in Early Atherosclerotic Lesions**

Luminal endothelial cells were positive for LOX-1 expression in most sections of the human carotid arteries showing early atherosclerotic lesions with subendothelial infiltrates (Figure 3C), although LOX-1 expression was not detectable in the unaffected human aortic endothelium (Figure 3A). Staining of an adjacent section with an anti-von Willebrand factor antibody (Figure 3E) and double-labeled immunostaining (Figure 3G) identified these LOX-1–positive cells as endothelial cells. In contrast, luminal endothelial cells in advanced atherosclerotic plaques were less frequently positive for LOX-1 expression (Figure 4E). LOX-1 expression in association with arterial luminal endothelial cells was present in 71.4% of samples with early lesions and in 33.3% of samples with advanced lesions (Table 1). These results suggest that LOX-1 expression in luminal endothelial cells seems to be upregulated, especially in the early stages of atherogenesis.

**LOX-1 Expression on Endothelial Cells of Intimal Neovascularure**

In advanced atherosclerotic plaques, neovascular formation in the intima was frequently observed. Of the segments with atherosclerotic plaques in this study, 18 of 21 samples (85.7%) had neovessel infiltration into the intima, which was identified by examining the hematoxylin- and eosin–stained sections. In these microvascular endothelial cells, expression of LOX-1 was often observed (Figure 5). LOX-1 expression in endothelial cells was present in 55.6% of advanced atherosclerotic plaques with intimal neovascularure (Table 1).

**LOX-1 was Expressed by Intimal Macrophages and Smooth Muscle Cells in Atherosclerotic Plaques**

In addition to the vascular endothelium, cells consisting of the neointima of advanced atherosclerotic plaques were also
Figure 3. Immunohistochemically stained human aorta both without visible atherosclerotic changes (A and B) and with early atherosclerotic lesions (C and G). A and B, Frozen serial sections of a nonlesional aorta were stained with anti-hLOX-1 monoclonal antibody (A, ×200) and anti-von Willebrand factor antibody (B, ×200). LOX-1 was not expressed in luminal endothelial cells of nonlesional aorta (A). Serial sections showed uniform staining in luminal surface of the aorta with anti-von Willebrand factor antibody, indicating that intact endothelial linings were preserved (B). C through F, Frozen serial sections of early atherosclerotic lesions were stained with anti-hLOX-1 monoclonal antibody (C, ×200; D, ×1000), anti-von Willebrand factor antibody (E, ×200), and nonimmune mouse IgG (F, ×200). LOX-1 was expressed in luminal endothelial cells of early atherosclerotic lesions with subendothelial infiltrates. Some nonendothelial cells in intima were also positive for anti-hLOX-1 monoclonal antibody. G, Double immunohistochemical staining with anti-bLOX-1 antibody (dark blue) and anti-von Willebrand factor antibody (red) in early atherosclerotic lesions (×1000). LOX-1 expression in luminal endothelial cells was confirmed in early atherosclerotic lesions.
Figure 4. Immunohistochemical staining of advanced atherosclerotic lesions. A through D, Frozen serial sections of advanced atherosclerotic lesions were stained with anti-hLOX-1 monoclonal antibody (A, ×100) and nonimmune mouse IgG (B, ×100). LOX-1 was expressed by nonendothelial cells in intima of advanced atherosclerotic lesions. Higher-power view of A showed that LOX-1–positive subendothelial cells included round cells consistent with morphology of macrophages (C, ×1000) and spindle-shaped cells consistent with morphology of smooth muscle cells (D, ×1000). E and F, Other serial sections of advanced atherosclerotic lesions were stained with anti-hLOX-1 monoclonal antibody (E, ×100) and anti-von Willebrand factor antibody (F, ×100). LOX-1 was not expressed by luminal endothelial cells stained with anti-von Willebrand factor antibody. Most intimal nonendothelial cells expressing LOX-1 were spindle-shaped, consistent with morphology of intimal smooth muscle cells. G and H, Double-labeled immunohistochemical staining with anti-hLOX-1 monoclonal antibody and antibodies for cell markers of advanced atherosclerotic lesions. Cell marker antibodies were visualized by alkaline phosphatase method and appeared red on slides, whereas the anti-hLOX-1 antibody was marked with avidin-biotin complex peroxidase technique and appeared dark blue. G, Double immunohistochemical staining with anti-hLOX-1 antibody...
Distribution of LOX-1 Expression in the Intima

<table>
<thead>
<tr>
<th>Distribution of LOX-1 in the Intima</th>
<th>No. of Positive Segments (%)</th>
<th>Early lesions</th>
<th>Advanced lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial Luminal Endothelial Cells</td>
<td>15/21 (71.4)</td>
<td>7/21 (33.3)</td>
<td></td>
</tr>
<tr>
<td>Intimal Neovascularture</td>
<td>NA</td>
<td>10/18 (55.6)</td>
<td></td>
</tr>
<tr>
<td>Intimal Nonendothelial Cells</td>
<td>21/21 (100)</td>
<td>21/21 (100)</td>
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</tr>
</tbody>
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Early lesions indicates samples distant from the center of the plaques. Early lesions, which showed some subendothelial infiltrates, did not contain a lipid core or fibrous cap. Advanced lesions indicates samples that consisted of fibrofatty plaques. A total of 21 early lesions and 21 advanced lesions were examined in this study. Intimal neovascularure was not seen in any samples of early lesions. Of the segments with advanced lesions in this study, 18 of 21 (85.7%) had neovessel infiltration into the intima.

Discussion

Ox-LDL may play crucial roles in the initiation and progression of atherosclerosis. Cellular uptake of Ox-LDL by macrophages and smooth muscle cells in the arterial intima seems to be involved in foam cell transformation and fatty streak formation. In addition, the endothelial activation elicited by Ox-LDL and its lipid constituents has been implicated in atherogenesis. LOX-1, a novel receptor for Ox-LDL, can be expressed mainly in vascular endothelial cells. LOX-1 expression in cultured smooth muscle cells has also been observed (N. Kume, MD, PhD, et al, unpublished data, 1999).

Ox-LDL receptors such as CD36 and MSR-A bind aged and apoptotic cells. Our recent studies have also shown that LOX-1 can bind and phagocytose aged and apoptotic cells. During atherogenesis, certain subpopulations of macrophages and smooth muscle cells can undergo apoptotic change. Therefore, LOX-1 may also be involved in the removal of apoptotic cells in the arterial wall and thus modulate atherosclerotic progression.

In summary, the present study provides evidence that LOX-1 is expressed in luminal endothelial cells, especially in the early stage of atherogenesis, and in intimal neovascular endothelial cells. More importantly, LOX-1 is highly expressed by macrophages and smooth muscle cells in the intima of human carotid atherosclerotic plaques. Our preliminary studies with hypercholesterolemic mice and rabbit models of atherosclerosis have revealed similar patterns of LOX-1 expression in endothelial cells, macrophages, and smooth muscle cells (data not shown), thus supporting the hypothesis that LOX-1 may play significant roles in both the foam cell transformation of macrophages and smooth muscle.
cells and endothelial activation. Further studies related to the regulatory mechanisms of LOX-1 expression in various cell types, as well as the pathophysiological consequences of Ox-LDL uptake through this novel Ox-LDL receptor, may provide new insights into the pathogenesis of atherosclerosis.

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References

Figure 5. Single-labeled immunohistochemical staining of advanced lesion with intimal neovascularure. Sections were stained with anti-hLOX-1 monoclonal antibody. Intimal neovascular endothelium and intimal nonendothelial cells expressed hLOX-1 (A, ×100; B, ×400).


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