Impact of Plasma Oxidized Low-Density Lipoprotein Removal on Atherosclerosis

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Background—Several clinical studies of statin therapy have demonstrated that lowering low-density lipoprotein (LDL) cholesterol prevents atherosclerotic progression and decreases cardiovascular mortality. In addition, oxidized LDL (oxLDL) is suggested to play roles in the formation and progression of atherosclerosis. However, whether lowering oxLDL alone, rather than total LDL, affects atherogenesis remains unclear.

Methods and Results—To clarify the atherogenic impact of oxLDL, lectin-like oxLDL receptor 1 (LOX-1), an oxLDL receptor, was expressed ectopically in the liver with adenovirus administration in apolipoprotein E–deficient mice at 46 weeks of age. Hepatic LOX-1 expression enhanced hepatic oxLDL uptake, indicating functional expression of LOX-1 in the liver. Although plasma total cholesterol, triglyceride, and LDL cholesterol levels were unaffected, plasma oxLDL was markedly and transiently decreased in LOX-1 mice. In controls, atherosclerotic lesions, detected by Oil Red O staining, were markedly increased (by 38%) during the 4-week period after adenoviral administration. In contrast, atherosclerotic progression was almost completely inhibited by hepatic LOX-1 expression. In addition, plasma monocyte chemotactic protein-1 and lipid peroxide levels were decreased, whereas adiponectin was increased, suggesting decreased systemic oxidative stress. Thus, LOX1 expressed in the livers of apolipoprotein E–deficient mice transiently removes oxLDL from circulating blood and possibly decreases systemic oxidative stress, resulting in complete prevention of atherosclerotic progression despite the persistence of severe LDL hypercholesterolemia and hypertriglyceridemia.

Conclusions—OxLDL has a major atherogenic impact, and oxLDL removal is a promising therapeutic strategy against atherosclerosis. (Circulation. 2008;118:75-83.)

Key Words: atherosclerosis ■ lipoproteins ■ oxidative stress ■ oxidized low-density lipoprotein

Atherosclerosis is the major factor underlying the increased incidence of coronary heart disease and central vascular disease in the industrialized world.1 Low-density lipoprotein (LDL) cholesterol is considered a major factor in atherosclerosis development.2 In this decade, several clinical studies of statin therapy have demonstrated the pivotal roles of lowering LDL cholesterol in preventing atherosclerotic progression and decreasing cardiovascular mortality.3

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Oxidative stress might play critical roles in many diseases. In particular, oxidation of LDL might be a key step in the development of atherosclerosis.4–6 Oxidized LDL (oxLDL) has been proposed to be involved in many atherogenic changes in the vascular wall such as expression of adhesion molecules, migration of macrophages and smooth muscle cells, release of chemokines,7 and impairment of endothelial nitric oxide production.8 Importantly, oxLDL is incorporated into macrophages via receptor-mediated endocytosis, leading to macrophage transformation into foam cells and thus the plaque formation of atherosclerotic lesions. Furthermore, oxLDL itself reportedly induces oxidative stress in endothelial cells, smooth muscle cells, and macrophages, resulting in a vicious cycle of atherogenic plaque formation.9 However,
the effectiveness of antioxidant therapy against atherosclerosis is controversial. In addition, antioxidants may inhibit not only oxLDL formation but also many other oxidation-sensitive pathways. Therefore, it is unclear that the antiatherogenic effects of antioxidants, if any, are due to inhibition of oxLDL formation. Thus, whether lowering oxLDL alone, rather than total LDL, affects atherogenesis remains to be elucidated. Therefore, to directly clarify the impact of oxLDL on the development of atherosclerosis, we designed a strategy for removing oxLDL from the circulation in a murine hypercholesterolemia model: apolipoprotein E (apoE)-deficient mice.

Several receptors for oxLDL have been identified in recent years. Lectin-like oxLDL receptor-1 (LOX-1) is one such receptor for oxLDL and is expressed in atherosclerotic lesions, including endothelial cells, macrophages, and smooth muscle cells, suggesting that LOX-1 actively incorporates oxLDL. Therefore, with the goal of removing oxLDL from the circulation, we ectopically expressed LOX-1 in the livers of apoE-deficient mice using an adenoviral gene transfer system.

Methods

Preparation of Recombinant Adenovirus

Recombinant adenovirus containing murine LOX-1 cDNA under the CAG promoter was constructed as described previously. A recombinant adenovirus bearing the bacterial β-galactosidase gene (lacZ) was used as a control.

Animals

Animal studies were conducted in accordance with the institutional guidelines for animal experiments at Tohoku University. apoE-deficient mice (The Jackson Laboratory, Bar Harbor, Me) were fed a standard chow. At 46 weeks of age, the baseline group of mice (n = 9) were killed to determine the extent of established lesions at this age. Adenoviruses were administered intravenously at a dose of 2 x 10^10 plaque-forming units to 46-week-old apoE-deficient mice.

Blood Analysis

Plasma total cholesterol, triglyceride, and adiponectin levels were determined as described previously. Plasma lipoproteins were analyzed by high-performance liquid chromatography with molecular sieve columns. The monococyte chemoattractant protein (MCP)-1 concentration was measured with an ELISA kit (R&D Systems, Minneapolis, Minn). Plasma alanine aminotransferase was measured with the transaminase test (C) (Skylight Biotech, Inc, Akita, Japan). The aortas were removed, cleaned, cut open with the luminal surface facing up, and then immersion fixed in 10% formalin in PBS. The inner aortic surfaces were stained with Oil Red O to visualize neutral lipid (cholesterol ester and triglycerides) accumulation for 25 minutes at room temperature. After rinsing with 60% isopropyl alcohol and distilled water, the Oil Red O–stained areas were quantified by Scion Image software analysis (Scion Corp, Frederick, Md) of the digitized microscopic images. Results were expressed as percentages of the lipid-accumulating lesion area to the total aortic area analyzed.

Hepatic Uptake of oxLDL

Human LDL (1.006<d<1.063 g/mL) was purified by ultracentrifugation and oxidized with CuSO₄ followed by labeling with fluorescent lipid (1,1′-dioctadecyl-3,3′,3′-tetramethylindocarbocyanine perchlorate [DiI]) as described previously. Thirty minutes after DiI-labeled oxLDL (12 μg) injection, murine livers were excised for the extraction of lipids and measurement of fluorescence as described previously. and livers of mice with nonlabeled oxLDL injection also were examined immunohistochemically. Fluorescent intensities of tissue lysates were measured with a fluorescence spectrophotometer (F-2000, Hitachi, Tokyo, Japan).

Histological Analysis

Mouse livers and aortas were removed and rinsed with saline. The tissues were fixed with 10% formalin and embedded in paraffin. Tissue sections were cut at a thickness of 4 μm and stained with hematoxylin and eosin. For immunohistochemistry, the streptavidin-biotin method was performed with a Histofine SAB-PO kit (Nichirei, Tokyo, Japan). Slides were deparaffinized and then autoclaved in citrate buffer for antigen retrieval, followed by incubation with antibodies to oxLDL (Calbiochem, San Diego, Calif), mac-3 (BD Bioscience, San Jose, Calif), and smooth muscle actin (Progen, Heidelberg, Germany). Finally, the slides were visualized by incubation with a substrate solution containing 3,3′-diaminobenzidine tetrahydrochloride.

Measurement of Atherosclerotic Lesions

The aortas were removed, cleaned, cut open with the luminal surface facing up, and then immersion fixed in 10% formalin in PBS. The inner aortic surfaces were stained with Oil Red O to visualize neutral lipid (cholesterol ester and triglycerides) accumulation for 25 minutes at room temperature. After rinsing with 60% isopropyl alcohol and distilled water, the Oil Red O-stained areas were quantified by Scion Image software analysis (Scion Corp, Frederick, Md) of the digitized microscopic images. Results were expressed as percentages of the lipid-accumulating lesion area to the total aortic area analyzed.

Quantitative Real-Time Polymerase Chain Reaction–Based Gene Expression

On day 5 after adenoviral administration, total RNAs in 0.1 g of the aortas and livers from 24-week-old lacZ and LOX-1 mice were isolated with ISOGEN (Wako Pure Chemical Co, Osaka, Japan), and cDNA was synthesized with a Cloned AMV First Strand Synthesis Kit (In vitrogen, Rockville, Md) using 5 μg total RNA. cDNA synthesized from total RNA was evaluated with real-time quantitative polymerase chain reaction (LightCycler Quick System 350S, Roche Diagnostics GmbH, Mannheim, Germany). The relative amount of mRNA was calculated with GAPDH as the invariant control. The primers used are described in Table I of the online Data Supplement.

Statistical Analysis

All data are expressed as mean ± SEM. All statistical analyses were performed with the Statistical Package for the Social Sciences version 13.0 (SPSS Japan Inc, Tokyo, Japan). Normality was tested with the Kolmogorov-Smirnov test. When data were normally distributed, the statistical significance of differences was assessed with the unpaired t test and 1-way ANOVA, followed by Tukey’s post hoc analyses. The Mann–Whitney U test was applied when data were not normally distributed. Repeated-measures ANOVA was used to assess changes in plasma oxLDL values measured serially in time between the 2 experimental groups. In all analyses, values of P < 0.05 were accepted as statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
Results

Adenoviruses encoding LOX-1 or LacZ cDNA were administered intravenously to apoE-deficient mice at 46 weeks of age. Mice of this age were chosen because atherosclerosis progresses dramatically during the period just before 1 year of age. As we reported previously, intravenous administration of recombinant adenoviruses results in selective transgene expression in the liver with no detectable expression in other tissues (data not shown). As shown in Figure 1A, administration of LOX-1 adenovirus induced LOX-1 expression in the livers of mice (LOX-1 mice), whereas no LOX-1 expression was detected in those of control mice given LacZ adenovirus (LacZ mice).

To examine hepatic uptake of oxLDL with ectopic expression of LOX-1, fluorescence-labeled oxLDL was injected intravenously, followed by measurement of fluorescence values in the liver with no detectable expression in other tissues (data not shown). As shown in Figure 1A, administration of LOX-1 adenovirus induced LOX-1 expression in the livers of mice (LOX-1 mice), whereas no LOX-1 expression was detected in those of control mice given LacZ adenovirus (LacZ mice).

To examine hepatic uptake of oxLDL with ectopic expression of LOX-1, fluorescence-labeled oxLDL was injected intravenously, followed by measurement of fluorescence values in the liver. Haptic fluorescence values were markedly increased in LOX-1 mice compared with LacZ mice (Figure 1B). In addition, 30 minutes after intravenous oxLDL injection, hepatic oxLDL deposition was demonstrated immunohistochemically with anti-oxLDL antibody (Figure 1C). Thus, LOX-1 was ectopically and functionally expressed in the liver as an oxLDL receptor.

On the other hand, levels of C-reactive protein, interleukin-1β, and tumor necrosis factor-α expression were not significantly altered in the liver (Figure 2B). Thus, LOX-1 ectopically expressed in the livers of apoE-deficient mice functionally incorporated oxLDL into hepatocytes, possibly increasing oxidative stress, but liver damage was apparently limited.

Next, plasma lipid parameters were measured. Hepatic LOX-1 expression did not significantly alter plasma total cholesterol or triglyceride levels (Figure 3A). In addition, cholesterol contents of the LDL and high-density lipoprotein fractions were not significantly altered in LOX-1 mice compared with those in LacZ mice (Figure 3B). In marked contrast, plasma oxLDL levels were dramatically decreased in LOX-1 mice for 2 weeks but returned to control levels by 3 weeks after adenoviral administration (Figure 3C), probably because of decreased adenovirus-mediated transgene expression in the liver after 2 weeks, as reported previously. Thus, adenovirus-mediated LOX-1 expression in the liver resulted in very transient and selective oxLDL removal from the circulation despite the persistence of severe hypercholesterolemia and hypertriglyceridemia induced by apoE deficiency.

To elucidate the effects of hepatic LOX-1 expression on atherosclerosis, the extents of atherosclerotic lesions were determined, as represented by the ratio of Oil Red O–positive areas to the entire aorta. Atherosclerotic lesions of LacZ mice increased markedly, by 38%, during the 4-week period after adenoviral administration (from 46 to 50 weeks of age) compared with those at baseline (46-week-old mice) (Figure 4A and 4B). In contrast, intriguingly, atherosclerotic lesion areas of LOX-1 mice were very similar to those at baseline.
and were significantly smaller than those of LacZ mice (Figure 4A and 4B). These findings indicate that hepatic LOX-1 expression completely inhibited the progression of aortic atherosclerosis during the 4-week period when atherosclerosis markedly progresses in control apoE-deficient mice. Thus, oxLDL removal from circulating blood, even transient, exerts striking antiatherogenic effects, indicating the enormous impact of oxLDL on atherosclerosis.

Next, we immunohistochemically examined macrophage and smooth muscle cell infiltration into the plaques. Mac-3 staining revealed that macrophage deposition in established plaque lesions did not differ between LacZ- and LOX-1 mice (Figure 5A and 5B). In contrast, in LOX-1 mice, smooth muscle actin–positive areas in plaques were larger, especially in the surface areas of plaque lesions, than in LacZ mice. In LacZ mice, smooth muscle actin–positive areas in plaques were significantly decreased compared with those at baseline, and these decrements were inhibited by hepatic LOX-1 expression (Figure 5C and 5D). These findings suggest oxLDL removal from the circulation to inhibit the increase in vulnerability that occurs during plaque progression.

Furthermore, plasma MCP-1 levels were significantly lower in LOX-1 mice than in LacZ mice (Figure 6A). In contrast, plasma levels of adiponectin, which is considered a protective molecule against vascular damage, were significantly higher in LOX-1 mice (Figure 6B). In addition, plasma lipid peroxide levels were markedly lower in LOX-1 mice (Figure 6C). Oxidative stress reportedly upregulates and downregulates MCP-1 in vascular cells and adiponectin in adipocytes, respectively. Taken together with the finding of decreased lipid peroxide levels, systemic oxidative stress is likely to be decreased in LOX-1 mice.

Then, we examined mRNA expressions of oxidative stress– and inflammation-related proteins in the aortas of 24-week-old LacZ and LOX-1 mice on day 5 after adenoviral administration. The antioxidant enzymes catalase, glutathione S-transferase, and manganese superoxide dismutase tended to be downregulated in the aortas of LOX-1 mice, although the differences did not reach statistical significance (Figure 7A). In addition, aortic expression of MCP-1, interleukin-6, and interleukin-1β was significantly decreased in LOX-1 mice (Figure 7B), indicating decreased oxidative stress and local inflammation.
inflammation in the aortas of LOX-1 mice. OxLDL itself reportedly induces oxidative stress in endothelial cells, smooth muscle cells, and macrophages, resulting in a vicious cycle of atherogenic plaque formation. Taken together, these results show that oxLDL removal from circulating blood may decrease systemic oxidative stress and inflammatory responses by blocking this vicious cycle, thereby exerting further beneficial effects against atherosclerosis.

**Discussion**

Several clinical studies have shown that lowering LDL cholesterol inhibits the progression of atherosclerosis. The
beneficial effects of statin therapy for reducing both atherogenic lipoproteins and cardiovascular mortality have been established in this decade. In the present study, despite not altering plasma LDL cholesterol levels, hepatic LOX-1 expression completely inhibited atherosclerotic progression. Thus, oxLDL, but not other LDL fractions, is likely to have a major impact on atherosclerosis development.

In recent reports, LDL cholesterol reduction was shown not only to inhibit coronary atheroma progression but also to induce regression of thoracic aortic plaques, as evaluated by magnetic resonance imaging. Moreover, aggressive lipid-lowering therapy, ie, LDL cholesterol removal, with LDL apheresis produced remarkable regression of coronary atherosclerotic plaques. Here, in LOX-1 mice, atherosclerotic progression was completely inhibited despite a very transient oxLDL decrease, suggesting not only preventive but also therapeutic effects of oxLDL removal. In addition, smooth muscle cells persisted in plaques, particularly in plaque

Figure 4. Hepatic LOX-1 expression completely inhibited atherosclerosis progression. A, Aortic atherosclerosis was evaluated as the Oil Red O–positive area. B, The Oil Red O–positive areas were quantified and expressed as percentages of the total aortic area in baseline (46-week-old) mice (gray bars; n=9), 50-week-old LacZ mice (white bars; n=13), and 50-week-old LOX-1 mice (black bars; n=13). Representative histological findings of the whole aorta are shown in A. Data are presented as mean±SE. **P<0.01.

Figure 5. Macrophages and smooth muscle cells in established plaques of LOX-1 mice. A, B, Macrophage depositions were determined immunohistochemically with anti-macrophage (mac-3) antibody (A), and positive areas were measured as the lesion percentage of whole plaques (B). C, D, Smooth muscle cell infiltration was determined immunohistochemically with anti–smooth muscle actin (SMA) antibody (C), and positive areas were measured as the lesion percentage of whole plaques (D). Representative histological findings of the plaque are shown in A and C.
surface areas of LOX-1 mice. OxLDL reportedly enhances apoptosis of smooth muscle cells.\textsuperscript{41–43} Therefore, removal of oxLDL from circulating blood may affect the characteristics of plaque lesions by inhibiting apoptosis of smooth muscle cells infiltrating plaque lesions.

Intriguingly, the plasma level of adiponectin, which prevents atherosclerosis development and improves insulin sensitivity, increased with hepatic LOX-1 expression. It was reported that systemic oxidative stress correlates negatively with plasma adiponectin in human subjects\textsuperscript{34} and decreases adiponectin expression in adipocytes.\textsuperscript{37} On the other hand, plasma MCP-1 levels were suppressed in LOX-1 mice. Oxidative stress may induce MCP-1 upregulation in vascular smooth muscle cells, leading to atherosclerosis formation by

Figure 6. Hepatic LOX-1 expression altered oxidative stress–related plasma parameters. Plasma monocyte chemotactic protein-1 (A), adiponectin (B), and lipid peroxide (C) levels were determined 2 weeks after adenoviral administration to LacZ mice (white bars) and LOX-1 mice (black bars; n=6 per group). Data are presented as mean±SE. *P<0.05.

Figure 7. Relative amounts of mRNAs of proteins related to oxidative stress or inflammation in the aorta. On day 5 after LacZ (white bars) or LOX-1 (black bars) adenovirus administration to 24-week-old apoE-deficient mice, relative amounts of mRNA of proteins related to oxidative stress (A) or inflammation (B) in the aorta were determined by quantitative RT-PCR and corrected with GAPDH as the internal standard (n=9 in LacZ mice, n=11 in LOX-1 mice). Data are presented as mean±SE. GST indicates glutathione S-transferase; MnSOD, manganese superoxide diamutase; and IL-6, interleukin-6. *P<0.05, **P<0.01.
promoting recruitment of inflammatory cells to the vessel wall.36 We cannot rule out the possibility that LOX-1 expressed in the liver scavenges other pro-oxidant molecules. However, it was reported that oxLDL itself potently induces oxidative stress.9 Therefore, oxLDL removal from circulating blood is likely to decrease systemic oxidative stress, resulting in adiponectin upregulation and MCP-1 downregulation, thereby exerting further beneficial effects against atherosclerosis.

The effectiveness of antioxidant therapy against atherosclerosis is controversial.10–15 In murine models, administration of antioxidants effectively reduces atherosclerosis.12 On the other hand, most clinical trials yielded negative results.15 This may be at least partly due to insufficient antioxidant effects of natural and synthetic compounds when administered to human subjects. In a randomized placebo-controlled study in healthy adults, daily administration of vitamin E at doses as high as 2 000 mg did not affect the breakdown of lipid peroxidation products despite a substantial increase in plasma vitamin E concentrations.45 In addition, high doses of these antioxidants reportedly have adverse effects,15 including the pro-oxidant effects of vitamin E at high doses.46 Therefore, clinical application of these antioxidants seems to be limited. Thus, the present results provide a potential new therapeutic target because the antiatherogenic effect was observed after transient lowering of oxLDL.

Conclusions

LOX1 expressed in the liver transiently removes oxLDL from circulating blood without altering total cholesterol or LDL cholesterol levels and is likely to decrease systemic oxidative stress, resulting in complete inhibition of atherosclerosis development in aged apoe-deficient mice. This study provides strong evidence of the major atherogenic impact of oxLDL. Removal of oxLDL, even transiently, is a promising therapeutic strategy for blocking the vicious cycle that leads to atherosclerosis.

Acknowledgments

We thank I. Sato, J. Fushimi, K. Kawamura, M. Aizawa, M. Hoshi, and T. Takasugi for technical support.

Sources of Funding

This work was supported by Grants in Aid for Scientific Research (B2, 15390282 to Dr Katagiri and 19591031 to Dr Ishigaki) from the Ministry of Education, Science, Sports and Culture of Japan and by a Grant in Aid for Scientific Research (H19-genome-005) from the Ministry of Health, Labor, and Welfare of Japan. This work also was supported by two 21st Century COE Programs (to Drs Oka and T. Takasugi for technical support.

We thank I. Sato, J. Fushimi, K. Kawamura, M. Aizawa, M. Hoshi, and T. Takasugi for technical support.

Disclosures

None.

References

A consensus has been reached that lowering plasma low-density lipoprotein (LDL) inhibits atherosclerosis progression. However, whether lowering plasma oxidized LDL (oxLDL) alone contributes to preventing atherosclerosis remains uncertain. The antiatherogenic effects of antioxidant therapy that may inhibit oxLDL formation are controversial because most clinical trials yielded negative results. Here, it has been shown that removal of oxLDL from the circulation has a very strong effect against atherosclerosis. In this study, lectin-like oxLDL receptor 1 (LOX-1), an oxLDL receptor, was expressed ectopically in the livers of apolipoprotein E–deficient mice (LOX-1 mice), using adenoviral gene transfer, to remove oxLDL from the circulation. Intriguingly, a transient decrease in plasma oxLDL, without affecting non-oxLDL cholesterol levels, completely inhibited atherosclerotic progression. Systemic oxidative stress was shown to be decreased in LOX-1 mice. Thus, the results of this study provide potential therapeutic targets for atherosclerosis, ie, treatments that would potently lower plasma oxLDL, including inhibition of oxLDL formation and removal of oxLDL from the circulation. These promising strategies may contribute to the prevention of not only atherosclerosis formation but also the development of acute coronary syndrome.
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Circulation. 2008;118:75-83; originally published online June 16, 2008;

doi: 10.1161/CIRCULATIONAHA.107.745174

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the

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### Sequences of Quantitative RT-PCR primers

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