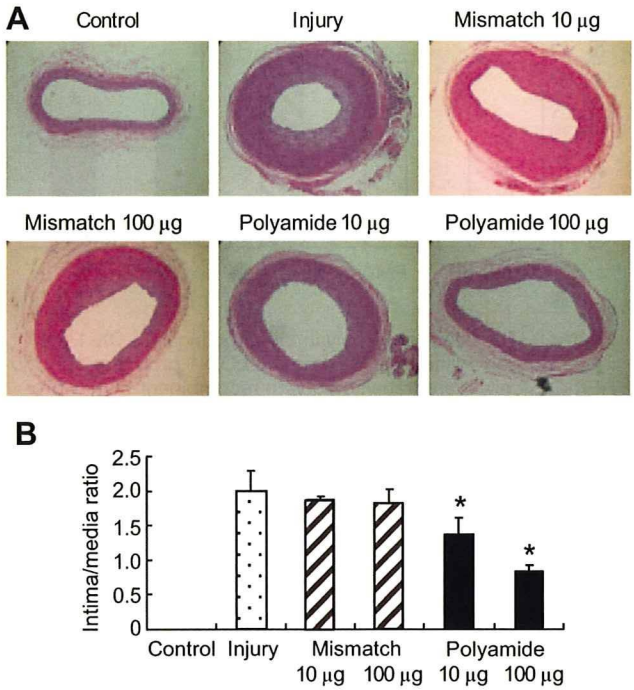

Figure 4. The effect of PI polyamide to LOX-1 on neointimal hyperplasia in rat carotid arteries at 21 days after injury. A, Hematoxylin-eosin staining of a cross section of the specimens treated without or with polyamide or mismatch polyamide. B, Intimal and medial cross-sectional areas of the arteries were determined with a computerized apparatus and National Institutes of Health Image software program. The data are the means±SEMs (n=4). *$P<0.05$ vs injury. Magnification: ×100.

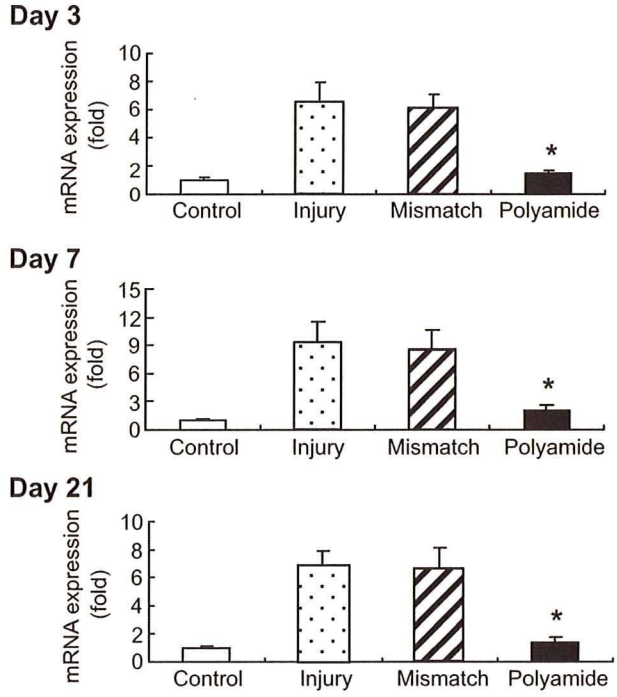

Figure 5. The effect of PI polyamide targeting rat LOX-1 promoter (PI polyamide) on the expression of LOX-1 mRNAs in rat carotid artery after balloon injury. A 2F embelectomy catheter with balloon was drawn toward the arteriotomy site 3 times. At 3, 7, and 21 days after the injury, arteries were incubated without (Injury) or with 100 μg of PI polyamide to LOX-1 (Polyamide) or mismatch polyamide (Mismatch) for 10 minutes. Total RNA was extracted, and LOX-1 mRNAs were evaluated by real-time PCR assay. The data are the ratios of LOX-1 to GAPDH mRNA abundances (means±SEMs; n=4). * $P<0.05$ vs injury.

LOX-1 significantly ($P<0.05$) reduced neointimal thickening by 31% and 58%, respectively, in comparison with the injury group. The mismatch polyamide did not affect the neointimal formation.

Effect of PI Polyamide to LOX-1 on Expression of LOX-1 in the Injured Artery

The expression of LOX-1 mRNA was significantly ($P<0.05$) higher in the injured artery than that in the noninjured artery. Treatments with PI polyamide to LOX-1 significantly ($P<0.05$) reduced the expression of LOX-1 mRNA in the artery at 3, 7, and 21 days after balloon injury. The treatments with mismatch polyamide did not affect the expression of LOX-1 mRNA in the artery (Figure 5). Immunofluorescence staining showed that LOX-1 was not detectable in the noninjured artery, whereas LOX-1 was markedly increased in the endothelial layer and midlayer smooth muscle in the injured artery at 21 days after injury. The treatment of PI polyamide to LOX-1 reduced the LOX-1 expression. The mismatch polyamide did not affect the expression of LOX-1 (Figure 6).

Effect of PI Polyamide to LOX-1 on Re-endothelialization in the Injured Artery

Immunohistochemistry of endothelial cells with anti–von Willebrand factor antibody in rat carotid artery at 21 days after balloon injury showed that endothelial cells were stained in the intimal surface after balloon injury. The treatment of PI polyamide to LOX-1 obviously enhanced the staining of the endothelial cells in the injured artery (Figure 6).

Effect of PI Polyamide to LOX-1 on the Expression of MCP-1, ICAM-1, and MMP-9 mRNAs in Injured Artery

The expression of MCP-1, ICAM-1, and MMP-9 mRNAs significantly ($P<0.05$) increased in the injured artery 3 days after injury in comparison with the noninjured artery. The treatment of PI polyamide to LOX-1 significantly ($P<0.05$) reduced the expression of these mRNAs. The mismatch polyamide did not affect the expression of these mRNAs (Figure 7).

Discussion

In the present study, we constructed deletion mutants and analyzed the rat LOX-1 promoter activity stimulated by PMA and found a transcription factor AP-1 binding site between bp −59 and −53 in the rat LOX-1 promoter. The site-directed mutation analysis further confirmed this positive regulatory element for the activation of LOX-1 promoter. These findings imply that, to interfere with this AP-1 binding site, PI polyamide to LOX-1 will suppress the LOX-1 gene activation and expression. For gene-specific targeting, the polyamide was then designed to target the sequence immediately adjacent to the binding site for AP-1 on the promoter. Synthetic PI polyamides have been shown to be cell permeable and able to

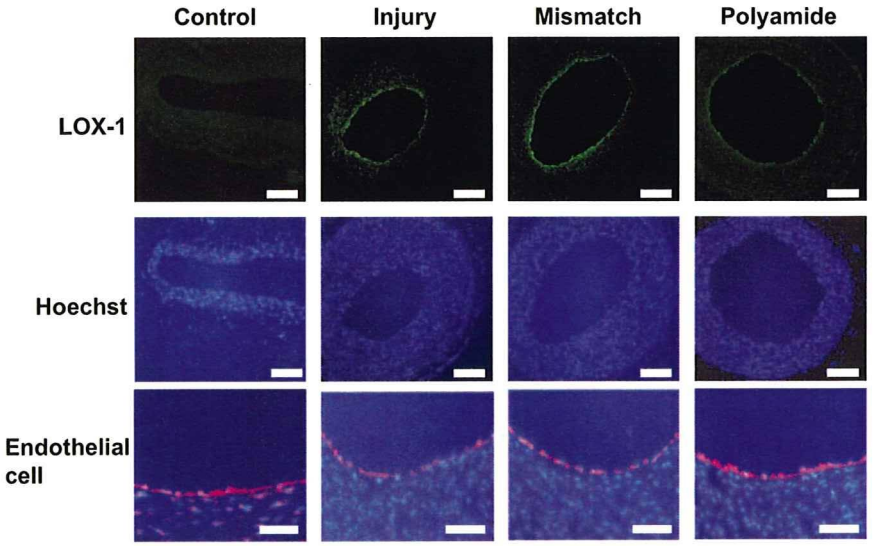

Figure 6. Effect of PI polyamide targeting rat LOX-1 promoter (PI polyamide to LOX-1) on the expression of LOX-1 protein and the re-endothelialization of rat carotid artery after balloon injury. A 2F embelectomy catheter with balloon was drawn toward the arteriotomy site 3 times. After the injury, arteries were incubated without (Injury) or with 100 μg of PI polyamide to LOX-1 (Polyamide) or mismatch polyamide (Mismatch) for 10 minutes. At 21 days after balloon injury the carotid arteries were removed and stained with anti–LOX-1 antibody (LOX-1) or anti–von Willebrand factor antibody (Endothelial cell), and incubated with fluorescein-conjugated respective secondary antibodies. The nuclei were stained with Hoechst 33342. Scale bar represents 200 μm for LOX-1 and Hoechst or 50 μm for endothelial cell.

inhibit the transcription of specific genes.[18] In our previous study, we also demonstrated that synthetic PI polyamides easily passed into the cells and then entered the nuclei of cells without any vector or delivery reagents to inhibit the expression of the target gene.[8,13] In this study, in a construct of plasmid, the polyamide significantly inhibited the LOX-1 promoter activity stimulated with PMA, thus suggesting that the synthetic PI polyamide to LOX-1 actually interfered with the AP-1 transcription factor-DNA interaction.

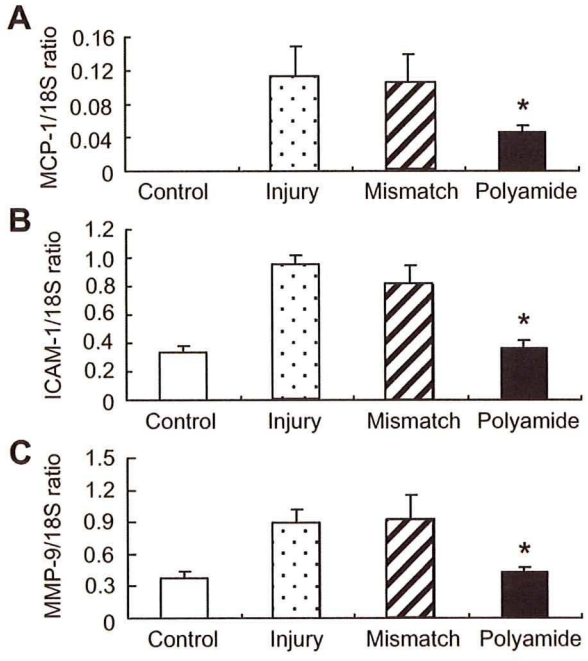

Figure 7. Effect of PI polyamide targeting rat LOX-1 promoter (PI polyamide to LOX-1) on the expression of MCP-1, ICAM-1, and MMP-9 mRNAs in rat carotid artery after balloon injury. A 2F embelectomy catheter with balloon was drawn toward the arteriotomy site 3 times. After the injury, arteries were incubated without (Injury) or with 100 μg of PI polyamide to LOX-1 (Polyamide) or a mismatch polyamide (Mismatch) for 10 minutes. At 3 days after balloon injury, total RNA was extracted and mRNA expression was evaluated by RT-PCR assay. The ratio of mRNA to 18S rRNA was evaluated. The data are the means±SEMs (n=4 to 6). *$P<0.05$ vs injury.

The designed PI polyamide to LOX-1 significantly inhibited the expression of LOX-1 mRNA and protein stimulated with PMA in cultured rat aortic endothelial cells, thus suggesting that PI polyamide to LOX-1 has the potential to control LOX-1 gene expression. LOX-1 has been reported to induce apoptosis of endothelial cells, which is associated with the atherosclerosis and restenosis of artery.[7,19] Because the endothelium has the ability to improve arterial injury, the denudation of the endothelium by coronary intervention may, thus, accelerate the occurrence of restenosis. Drug-eluting stents (DESs) have been shown to be effective for preventing in-stent restenosis. The sirolimus-coated DESs can prevent in-stent restenosis by inducing the complete inhibition of vascular smooth muscle cell hyperplasia by its effect on cell cycle arrest.[20] However, complications such as subacute thrombosis or late thrombosis have been reported recently in patients implanted with a sirolimus-coated DES.[21] Sirolimus prevents re-endothelialization of the inner side of the metal stent, which may cause late thrombosis. These complications have led to the development of second-generation DESs that do not induce late thrombosis. In the present study, the rapid regeneration of endothelial cells may appear to contribute to the suppression of intimal hyperplasia after treatment with PI polyamide to LOX-1. The preservation of the endothelium by PI polyamide to LOX-1 is, thus, considered to be very advantageous for DESs to prevent both restenosis and late thrombosis.

In the present study, FITC-labeled PI polyamide to LOX-1 was well distributed into the wall of the carotid artery and strongly bound the cell nucleus without any vectors after injury. Nucleic acid medicines, such as antisense DNA, ribozymes, and decoy, have been developed as gene-silencing agents. Decoys, in particular, inhibit the binding of target transcription factors in a manner similar to polyamides. However, because these agents tend to easily degrade when coming into contact with nucleases, they require drug-delivery systems for sufficient distribution into organs. In contrast, PI polyamides are completely resistant to nucleases and can be delivered into organs without delivery systems.

In the present study, the expression of LOX-1 was low in the noninjury artery, whereas the expression of LOX-1 was significantly increased after balloon injury. PI polyamide to LOX-1 effectively inhibited LOX-1 expression in the injured artery and attenuated the neointimal formation of the artery after injury. LOX-1 has been reported to be expressed in atheromatous lesions and is involved in neointimal hyperplasia after vascular injury.[7] Therefore, LOX-1 is a target for the treatment of restenosis, and the polyamide against LOX-1 may be an effective approach to inhibit restenosis.

LOX-1 is a main receptor for ox-LDL. Ox-LDL plays a role in the initiation and progression of atherosclerosis via LOX-1.[22] Other than ox-LDL, LOX-1 binds multiple classes of ligands that are implicated in the pathogenesis of atherosclerosis by the apoptosis of cells and the activation of platelets.[23] A significant number of apoptotic cells have been reported to be present in restenotic lesions after balloon injury,[24] thus implying that several factors presented after arterial injury may interact with and activate LOX-1. The activation of LOX-1 may, therefore, increase superoxide generation, reduce the production of NO, induce MCP-1, and increase leukocyte adhesiveness.[25] Hinagata et al[7] demonstrated that the inhibition of LOX-1 with anti–LOX-1 antibody attenuated oxidative stress in the neointima of the rat injured artery. In addition, antioxidative agents have been reported to inhibit the neointimal hyperplasia in normocholesterolemic rabbits and pigs.[26] In the present study, the expression of MCP-1, ICAM-1, and MMP-9 mRNAs was markedly increased in the injured artery, which was significantly decreased with treatments of PI polyamide to LOX-1, suggesting that increases in these molecules are associated with the induction of LOX-1 in the injured artery. MCP-1 is a potent chemotactic factor of monocytes[25] and is produced by activated vascular smooth muscle cells or other type of cells.[27] Antisense oligodeoxynucleotides to LOX-1 inhibit MCP-1 and monocyte adhesion.[28] The inhibition of MCP-1 results in a significant attenuation of neointimal hyperplasia.[29] MMP-9 is upregulated after angioplasty and involved in regulating the proliferation and migration of vascular smooth muscle cells, which are crucial steps for intimal hyperplasia.[30] Therefore, the designed PI polyamide to LOX-1 may attenuate intimal hyperplasia through cellular adhesion to the injured artery. This might partially explain the beneficial effects of the PI polyamide targeting LOX-1 on the suppression of neointimal hyperplasia.

In conclusion, the synthetic PI polyamide to LOX-1 potentially suppressed the LOX-1 promoter activity. PI polyamide to LOX-1 was delivered in midlayer smooth muscle of an injured artery without delivery reagents and significantly inhibited the intimal hyperplasia with the downregulation of MCP-1, ICAM-1, and MMP-9 and re-endothelialization in the injured artery. PI polyamide to LOX-1 is a potentially effective agent for the treatment of in-stent restenosis and will be a candidate agent for the development of next-generation DES.

Perspectives

Because polyamides can be readily designed and synthesized to target any gene, they are, therefore, expected to become important gene-silencing agents in the postgenome era. PI polyamide to LOX-1 is, therefore, considered to be a feasible gene silencing agent for the prevention of in-stent restenosis of the coronary artery as a next generation agent for DES.

Sources of Funding

This work was supported in part by a grant-in-aid for the Nihon University Multidisciplinary Research Grant for 2006 and the Academic Frontier Project for 2006 Project for Private Universities.

Disclosures

None.

References

1. Fischman DL, Leon MB, Baim DS, Schatz RA, Savage MP, Penn I, Detre K, Veltri L, Ricci D, Nobuyoshi M, Cleman M, Heuser R, Almond D, Teirstein PS, Fish RD, Colombo A, Brinker J, Moses J, Shaknovich A, Hirshfeld J, Bailey S, Ellis S, Rake R, Goldberg S. A randomized comparison of coronary-stent placement and balloon angioplasty in the treatment of coronary artery disease. Stent Restenosis Study Investigators. *N Engl J Med.* 1994;331:496–501.
2. Serruys PW, de Jaegere P, Kiemeneij F, Macaya C, Rutsch W, Heyndrickx G, Emanuelsson H, Marco J, Legrand V, Materne P, Belardi J, Sigwart U, Colombo A, Goy JJ, van den Heuvel P, Delcan J, Morel M. A comparison of balloon-expandable-stent implantation with balloon angioplasty in patients with coronary artery disease. Benestent Study Group. *N Engl J Med.* 1994;331:489–495.
3. Virmani R, Farb A. Pathology of in-stent restenosis. *Curr Opin Lipidol.* 1999;10:499–506.
4. Tardif JC, Grégoire J, L'Allier PL. Prevention of restenosis with antioxidants: mechanisms and implications. *Am J Cardiovasc Drugs.* 2002; 2:323–334.
5. Erl W, Weber PC, Weber C. Monocytic cell adhesion to endothelial cells stimulated by oxidized low density lipoprotein is mediated by distinct endothelial ligands. *Atherosclerosis.* 1998;136:297–303.
6. Sawamura T, Kume N, Aoyama T, Moriwaki H, Hoshikawa H, Aiba Y, Tanaka T, Miwa S, Katsura Y, Kita T, Masaki T. An endothelial receptor for oxidized low-density lipoprotein. *Nature.* 1997;386:73–77.
7. Hinagata J, Kakutani M, Fujii T, Naruko T, Inoue N, Fujita Y, Mehta JL, Ueda M, Sawamura T. Oxidized LDL receptor LOX-1 is involved in neointimal hyperplasia after balloon arterial injury in a rat model. *Cardiovasc Res.* 2006;69:263–271.
8. Lai YM, Fukuda N, Ueno T, Matsuda H, Saito S, Matsumoto K, Ayame H, Bando T, Sugiyama H, Mugishima H, Serie K. Synthetic pyrrole-imidazole polyamide inhibits expression of the human transforming growth factor-beta1 gene. *J Pharmacol Exp Ther.* 2005;315:571–575.
9. Trauger JW, Baird EE, Dervan PB. Recognition of DNA by designed ligands at subnanomolar concentrations. *Nature.* 1996;382:559–561.
10. White S, Baird EE, Dervan PB. On the pairing rules for recognition in the minor groove of DNA by pyrrole-imidazole polyamides. *Chem Biol.* 1997;4:569–578.
11. Fujimoto K, Iida H, Kawakami M, Bando T, Tao ZF, Sugiyama H. Sequence-specific protection of plasmid DNA from restriction endonuclease hydrolysis by pyrrole-imidazole-cyclopropapyrroloindole conjugates. *Nucleic Acids Res.* 2002;30:3748–3753.
12. Murty MS, Sugiyama H. Biology of N-methylpyrrole-N-methylimidazole hairpin polyamide. *Biol Pharm Bull.* 2004;27:468–474.
13. Matsuda H, Fukuda N, Ueno T, Tahira Y, Ayame H, Zhang W, Bando T, Sugiyama H, Saito S, Matsumoto K, Mugishima H, Serie K. Development of gene silencing pyrrole-imidazole polyamide targeting the TGF-β1 promoter for treatment of progressive renal diseases. *J Am Soc Nephrol.* 2006;17:422–432.
14. Bando T, Narita A, Saito I, Sugiyama H. Molecular design of a pyrrole-imidazole hairpin polyamide for effective DNA alkylation. *Chemistry.* 2002;8:4781–4790.
15. Tahira Y, Fukuda N, Endo M, Suzuki R, Ikeda Y, Takagi H, Matsumoto K, Kanmatsuse K. Transforming growth factor-beta expression in cardiovascular organs in stroke-prone spontaneously hypertensive rats with the development of hypertension. *Hypertens Res.* 2002;25:911–918.
16. Clowes AW, Reidy MA, Clowes MM. Kinetics of cellular proliferation after arterial injury: I. Smooth muscle growth in the absence of endothelium. *Lab Invest.* 1983;49:327–333.

17. Ando H, Fukuda N, Kotani M, Yokoyama S, Kunimoto S, Matsumoto K, Saito S, Kanmatsuse K, Mugishima H. Chimeric DNA–RNA hammerhead ribozyme targeting transforming growth factor-β1 mRNA inhibits neointima formation in rat carotid artery after balloon injury. *Eur J Pharmacol*. 2004;483:207–214.
18. Gottesfeld JM, Neely L, Trauger JW, Baird EE, Dervan PB. Regulation of gene expression by small molecules. *Nature*. 1997;387:202–205.
19. Chen M, Masaki T, Sawamura T. LOX-1, the receptor for oxidized low-density lipoprotein identified from endothelial cells: implications in endothelial dysfunction and atherosclerosis. *Pharmacol Ther*. 2002;95:89–100.
20. Regar E, Serruys PW, Bode C, Holubarsch C, Guermonprez JL, Wijns W, Bartorelli A, Constantini C, Degertekin M, Tanabe K, Disco C, Wuelfert E, Morice MC. Angiographic findings of the multicenter Randomized Study With the Sirolimus-Eluting Bx Velocity Balloon-Expandable Stent (RAVEL): sirolimus-eluting stents inhibit restenosis irrespective of the vessel size. *Circulation*. 2002;106:1949–1956.
21. Iakovou I, Sangiorgi GM, Stankovic G, Corvaja N, Michev I, Chieffo A, Rogacka R, Vitrella G, Airoldi F, Colombo A. Effectiveness of sirolimus-eluting stent implantation for treatment of in-stent restenosis after brachytherapy failure. *Am J Cardiol*. 2004;94:351–354.
22. Li D, Mehta JL. Upregulation of endothelial receptor for oxidized LDL (LOX-1) by oxidized LDL and implications in apoptosis of human coronary artery endothelial cells: evidence from use of antisense LOX-1 mRNA and chemical inhibitors. *Arterioscler Thromb Vasc Biol*. 2000;20:1116–1122.
23. Oka K, Sawamura T, Kikuta K, Itokawa S, Kume N, Kita T, Masaki T. Lectin-like oxidized low-density lipoprotein receptor 1 mediates phagocytosis of aged/apoptotic cells in endothelial cells. *Proc Natl Acad Sci USA*. 1998;95:9535–9540.
24. Isner JM, Kearney M, Bortmann S, Passeri J. Apoptosis in human atherosclerosis and restenosis. *Circulation*. 1995;91:2703–2711.
25. Li D, Mehta JL. Antisense to LOX-1 inhibits oxidized LDL-mediated upregulation of monocyte chemoattractant protein-1 and monocyte adhesion to human coronary artery endothelial cells. *Circulation*. 2000;101:2889–2895.
26. Tanaka K, Hayashi K, Shingu T, Kuga Y, Nomura K, Kajiyama G. Probucol inhibits neointimal formation in carotid arteries of normocholesterolemic rabbits and the proliferation of cultured rabbit vascular smooth muscle cells. *Cardiovasc Drugs Ther*. 1998;12:19–28.
27. Valente AJ, Graves DT, Vialle-Valentin CE, Delgado R, Schwartz CJ. Purification of a monocyte chemotactic factor secreted by nonhuman primate vascular cells in culture. *Biochemistry*. 1988;27:4162–4168.
28. Furukawa Y, Matsumori A, Ohashi N, Shioi T, Ono K, Harada A, Matsushima K Sasayama S. Anti-monocyte chemoattractant protein-1/monocyte chemotactic and activating factor antibody inhibits neointimal hyperplasia in injured rat carotid arteries. *Circ Res*. 1999;84:306–314.
29. Yukihiro H, Ikeda U, Katsuki T, Mizuno O, Fujikawa H, Shimada K. Matrix metalloproteinase expression in the coronary circulation induced by coronary angioplasty. *Atherosclerosis*. 2001;161:185–192.
30. Robinson EA, Yoshimura T, Leonard EJ, Tanaka S, Griffin PR, Shabanowitz J, Hunt DF, Appella E. Complete amino acid sequence of a human monocyte chemoattractant, a putative mediator of cellular immune reactions. *Proc Natl Acad Sci U S A*. 1989;86:1850–1854.

Impact of Plasma Oxidized Low-Density Lipoprotein Removal on Atherosclerosis

Yasushi Ishigaki, MD, PhD*; Hideki Katagiri, MD, PhD*; Junhong Gao, MD, PhD*; Tetsuya Yamada, MD, PhD; Junta Imai, MD, PhD; Kenji Uno, MD, PhD; Yutaka Hasegawa, MD, PhD; Keizo Kaneko, MD; Takehide Ogihara, MD, PhD; Hisamitsu Ishihara, MD, PhD; Yuko Sato, PhD; Kenji Takikawa, BA; Norihisa Nishimichi, PhD; Haruo Matsuda, DVM, PhD; Tatsuya Sawamura, MD, PhD; Yoshitomo Oka, MD, PhD

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]

Clinical Perspective p 83

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,

Received October 9, 2007; accepted April 18, 2008.

From the Division of Molecular Metabolism and Diabetes (Y.I., J.G., T.Y., J.I., Y.H., K.K., H.I., Y.O.) and Division of Advanced Therapeutics for Metabolic Diseases, Center for Translational and Advanced Animal Research (H.K., K.U., K.K., T.O.), Tohoku University Graduate School of Medicine, Sendai; Department of Vascular Physiology, National Cardiovascular Center Research Institute, Osaka (Y.S., T.S.); and Laboratory of Immunobiology, Department of Molecular and Applied Biosciences, Graduate School of Biosphere Science, Hiroshima University, Hiroshima (K.T., N.N., H.M.), Japan.

*Drs Ishigaki, Katagiri, and Gao contributed equally to this work.

The online Data Supplement, which contains a table, can be found with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.107.745174/DC1.

Correspondence to Hideki Katagiri, MD, PhD, Division of Advanced Therapeutics for Metabolic Diseases, Center for Translational and Advanced Animal Research, Tohoku University Graduate School of Medicine, 2-1 Seiryo-Machi, Aoba-Ku, Sendai 980-8575, Japan. E-mail katagiri@mail.tains.tohoku.ac.jp

Circulation **is available at http://circ.ahajournals.org** **DOI: 10.1161/CIRCULATIONAHA.107.745174**

the effectiveness of antioxidant therapy against atherosclerosis is controversial.[10–15] 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.[16–20] Lectin-like oxLDL receptor-1 (LOX-1) is one such receptor for oxLDL[21] and is expressed in atherosclerotic lesions, including endothelial cells, macrophages, and smooth muscle cells,[22] 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.[23,24] A recombinant adenovirus bearing the bacterial β-galactosidase gene (*lacZ*) was used as a control.[25]

Animals

Animal studies were conducted in accordance with the institutional guidelines for animal experiments at Tohoku University. ApoE-deficient mice[26] (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 2×10^8 plaque-forming units to 46-week-old apoE-deficient mice.

Blood Analysis

Plasma total cholesterol, triglyceride, and adiponectin levels were determined as described previously.[27] Plasma lipoproteins were analyzed by high-performance liquid chromatography with molecular sieve columns[28] (Skylight Biotech, Inc, Akita, Japan). The monocyte 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 (Wako Pure Chemicals, Osaka, Japan). Plasma levels of lipid peroxides were quantified with an LPO determiner (Kyowa Medex, Tokyo, Japan). OxLDL levels were measured with a sandwich ELISA. Murine plasma samples were applied to a plate coated with human soluble LOX-1 protein and detected with anti-apoB as the first antibody and goat anti-chicken IgG (H+L) (KPL, Inc, Gaithersburg, Md) as the detecting antibody. The reaction was developed with a tetramethylbenzidine peroxidase EIA substrate kit (Bio-Rad Laboratories, Hercules, Calif), and absorbance was measured at 450 nm.

Immunoblotting

Hepatic protein extracts (250 μg total protein) were boiled in Laemmli buffer containing 10 mmol/L dithiothreitol, subjected to SDS-PAGE, and transferred onto nitrocellulose filters. The filters were incubated with the murine LOX-1 antibody and then with anti-goat immunoglobulin G coupled to horseradish peroxidase. The immunoblots were visualized with an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK).

Hepatic Uptake of oxLDL

Human LDL ($1.006<d<1.063$ g/mL) was purified by ultracentrifugation and oxidized with $CuSO_4$,[29] followed by labeling with fluorescent lipid (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate [DiI]) as described previously.[30] 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,[31] 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).[32] 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 (cholesteryl 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 (Invitrogen, 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.

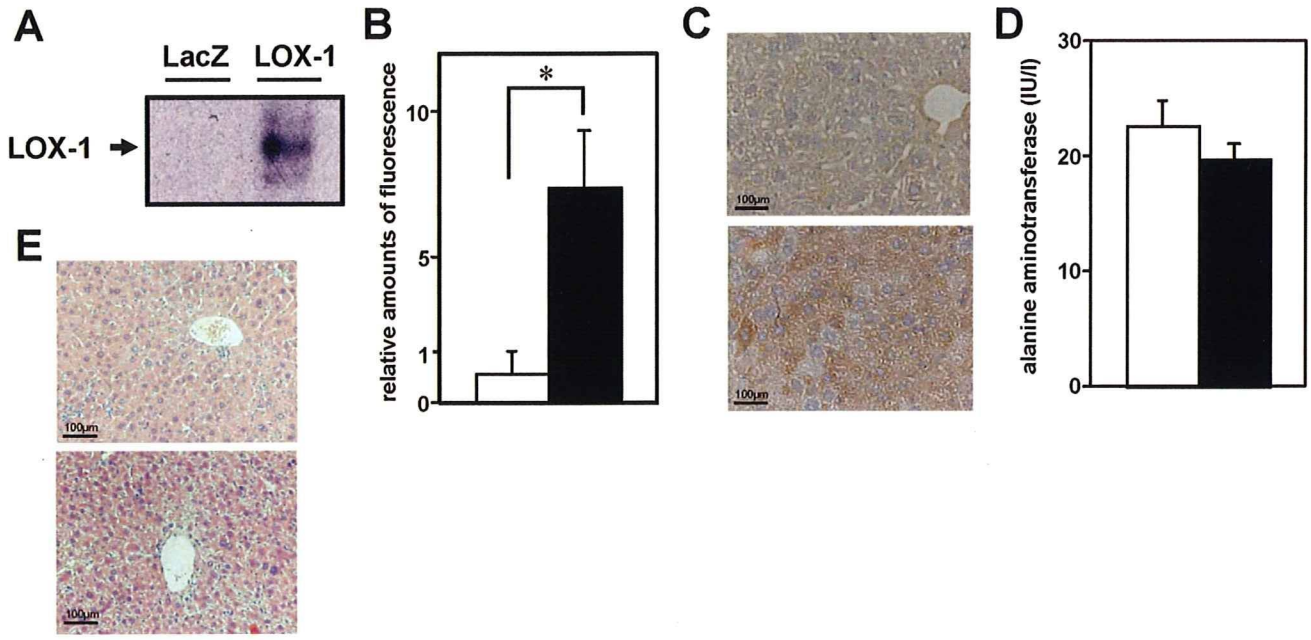

Figure 1. LOX-1 was ectopically and functionally expressed in the liver as an oxLDL receptor. A, Liver extracts were immunoblotted with anti–LOX-1 antibody 5 days after adenoviral administration. B, Mouse livers were removed 30 minutes after intravenous injection of DiI-labeled oxLDL, followed by measurement of fluorescent values in the livers of LacZ mice (white bars) and LOX-1 mice (black bars; n=5 per group). C, The livers of LacZ mice (top) and LOX-1 mice (bottom) were removed 30 minutes after intravenous oxLDL injection, followed by staining of hepatic sections with anti-oxLDL antibody. D, Plasma alanine aminotransferase levels were determined 5 days after adenoviral administration to LacZ mice (white bars) and LOX-1 mice (black bars; n=6 per group). E, The livers of LacZ mice (top) and LOX-1 mice (bottom) were stained with hematoxylin and eosin 4 weeks after adenoviral administration. B, D, Data are presented as mean±SE. *$P<0.05$.

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.[33] As we reported previously,[34] 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. Hepatic 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, plasma alanine aminotransferase levels were similar in LacZ- and LOX-1 mice (Figure 1D). In addition, histological analyses revealed no apparent infiltration or structural changes in the livers of LOX-1 mice (Figure 1E). However, hepatic expression of antioxidant enzymes, ie, catalase and glutathione S-transferase, was significantly upregulated (Figure 2A), suggesting increased oxidative stress in hepatocytes. 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.[27] 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

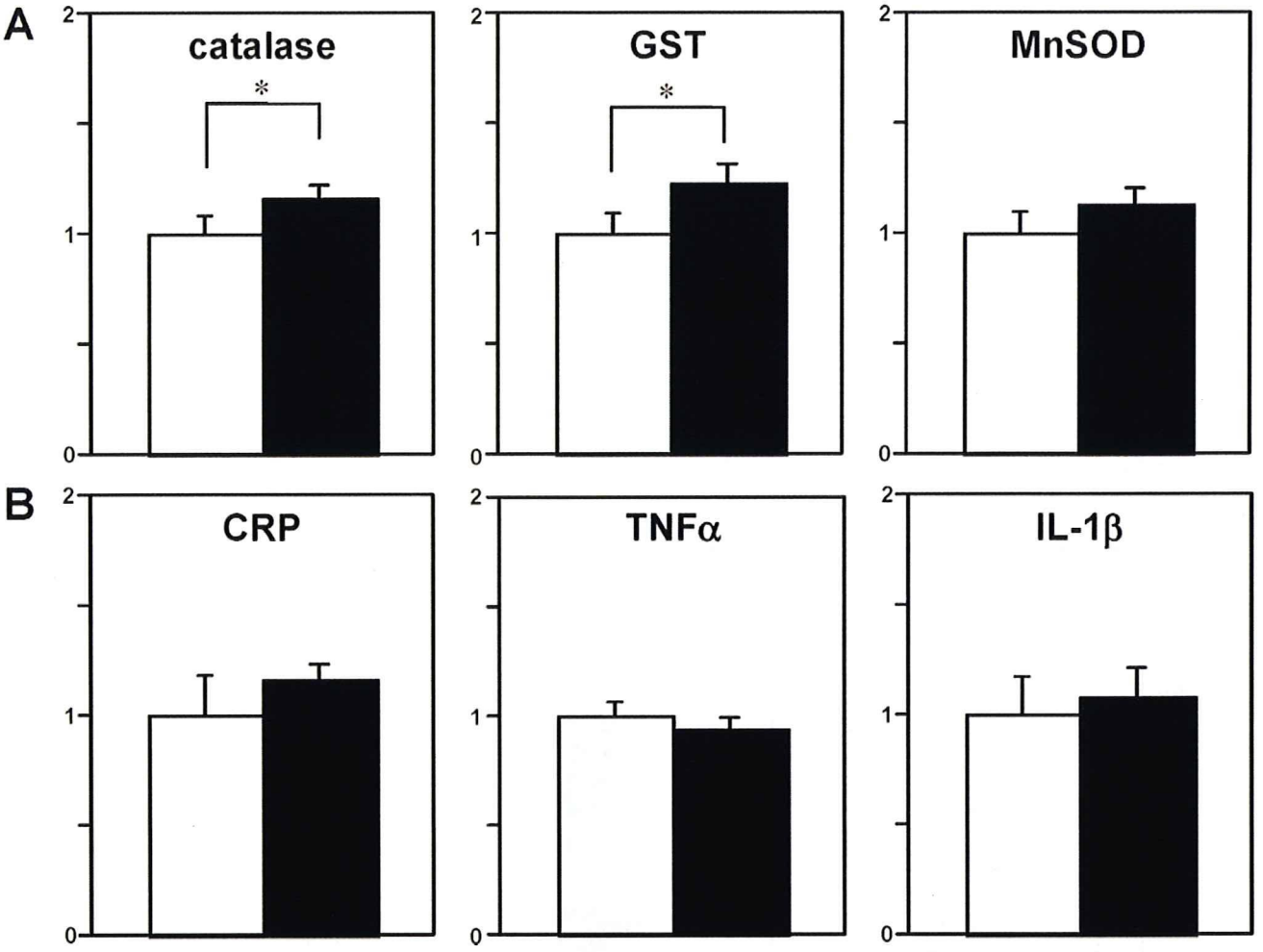

Figure 2. Relative amounts of mRNAs of proteins related to oxidative stress or inflammation in the liver. On day 5 after LacZ (white bars) or LOX-1 (black bars) adenovirus administration to 24-week-old apoE-deficient mice, relative amounts of mRNA of proteins related to oxidative stress (A) or inflammation (B) in the liver were determined by quantitative real-time polymerase chain reaction 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 dismutase; CRP, C-reactive protein; TNFα, tumor necrosis factor-α; and IL-1β, interleukin 1β. *$P<0.05$.

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,[35] 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[36] and adiponectin in adipocytes,[37] 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

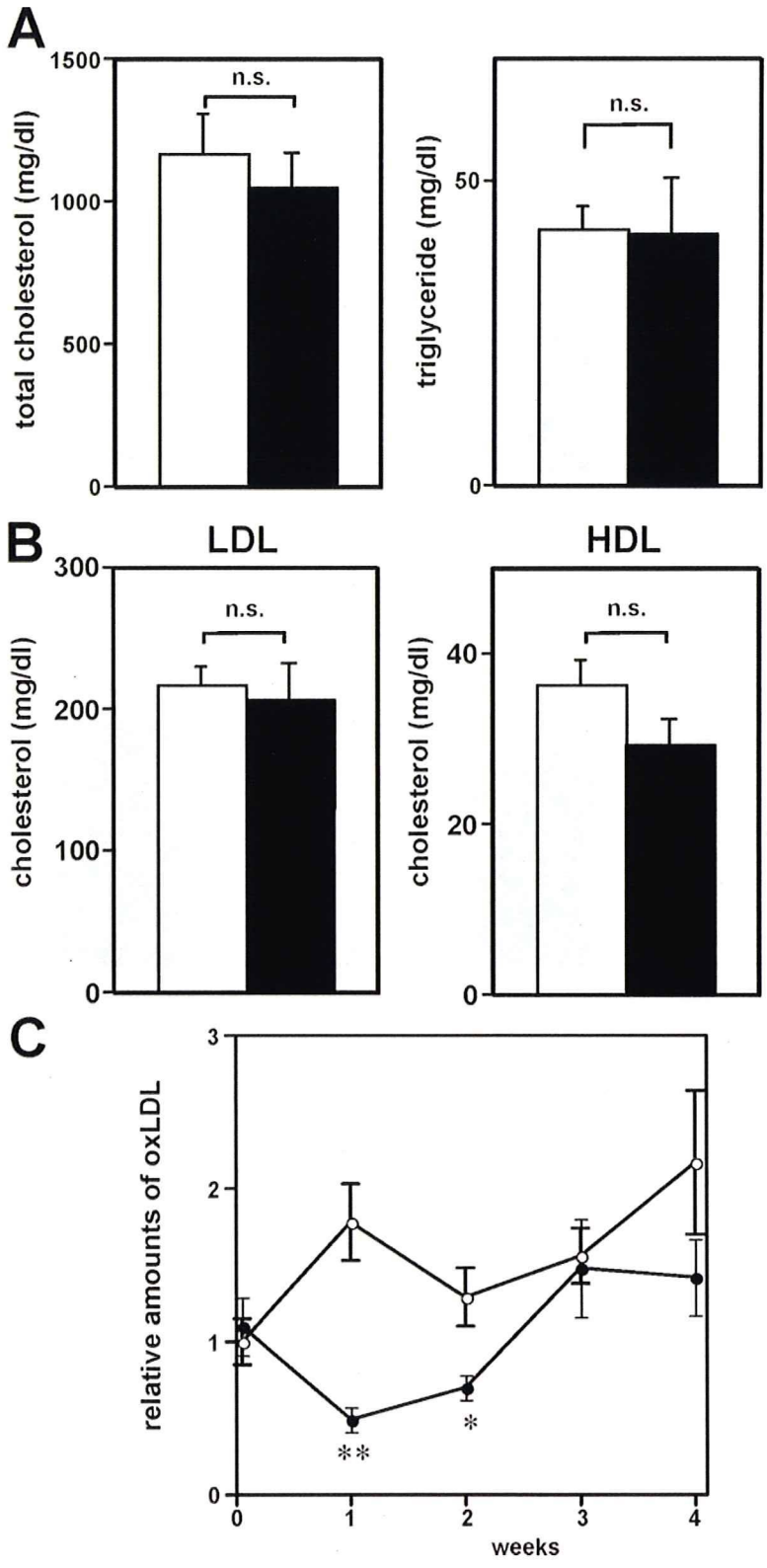

Figure 3. Hepatic LOX-1 expression transiently decreased plasma oxLDL without altering total cholesterol, triglyceride, or LDL cholesterol levels. A, Plasma total cholesterol (left) and triglyceride (right) levels of LacZ mice (white bars) and LOX-1 mice (black bars) were measured after a 10-hour fast 2 weeks after adenoviral administration (n=5 per group). B, Plasma samples (20 μL) from each mouse were separated and analyzed by high-performance liquid chromatography. Cholesterol contents of LDL and high-density lipoprotein (HDL) fractions were determined in LacZ mice (white bars) and LOX-1 mice after a 10-hour fast 2 weeks after adenoviral administration (black bars; n=3 per group). C, Plasma oxLDL levels were determined weekly until 4 weeks after adenoviral administration in LacZ mice (○) and LOX-1 mice (●; n=6 per group). Data are presented as mean±SE. $^{*}P<0.05$, $^{**}P<0.01$.

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.[9] 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.[3] The

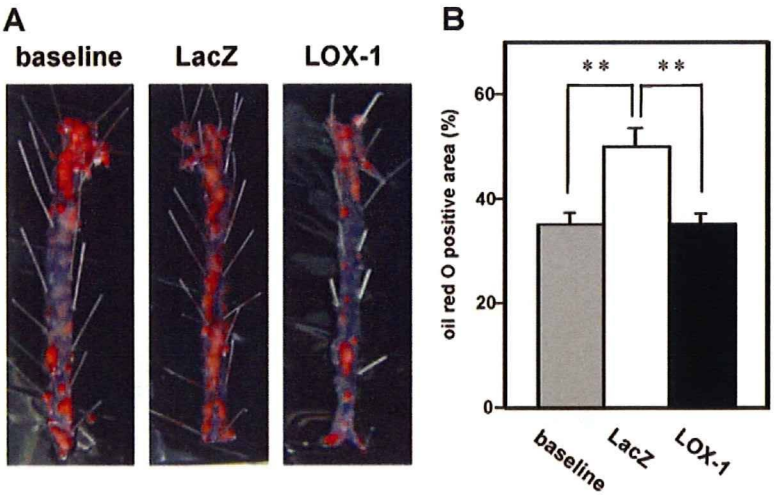

Figure 4. Hepatic LOX-1 expression completely inhibited atherosclerosis progression. A, Aortic atherosclerosis was evaluated as the Oil Red O–positive area. B, The Oil Red O–positive areas were quantified and expressed as percentages of the total aortic area in baseline (46-week-old) mice (gray bars; 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. $^{**}P<0.01$.

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[38] but also to induce regression of thoracic aortic plaques, as evaluated by magnetic resonance imaging.[39] Moreover, aggressive lipid-lowering therapy, ie, LDL cholesterol removal, with LDL apheresis produced remarkable regression of coronary atherosclerotic plaques.[40] 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

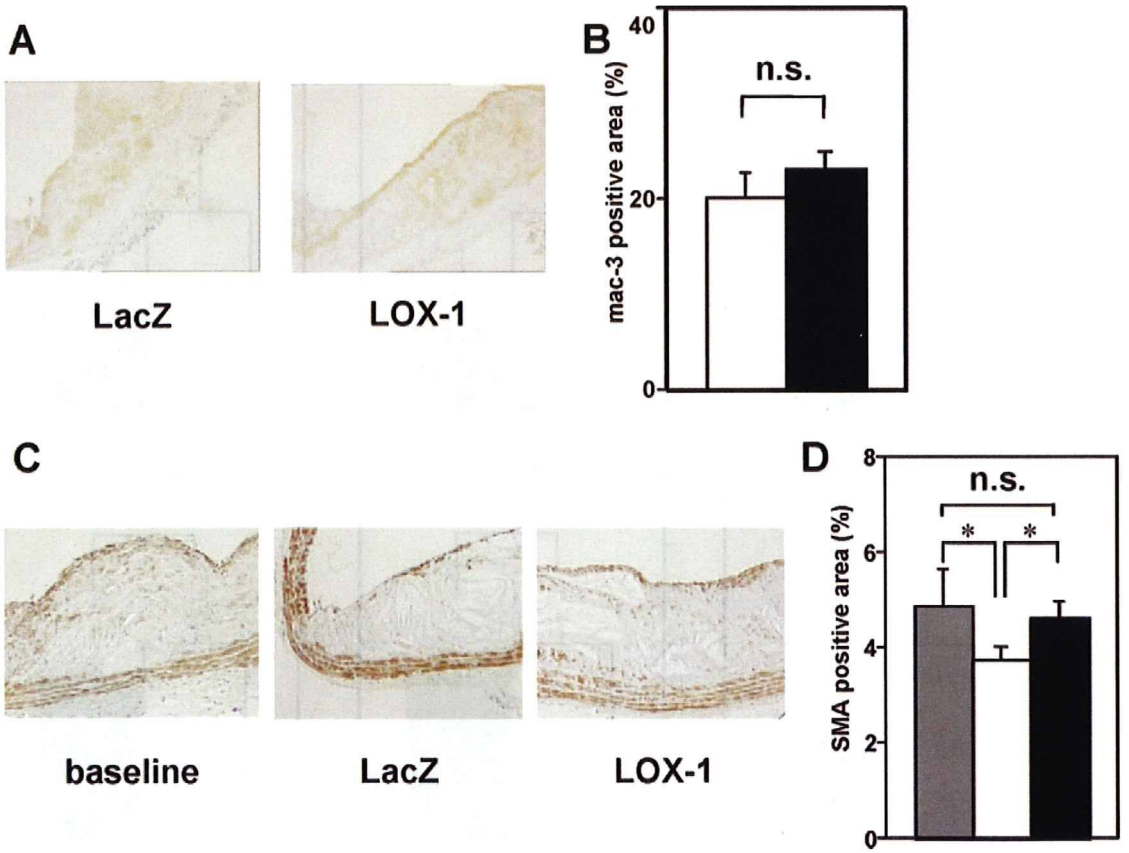

Figure 5. Macrophages and smooth muscle cells in established plaques of LOX-1 mice. A, B, Macrophage depositions were determined immunohistochemically with anti-macrophage (mac-3) antibody (A), and positive areas were measured as the lesion percentage of whole plaques (B). C, D, Smooth muscle cell infiltration 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.

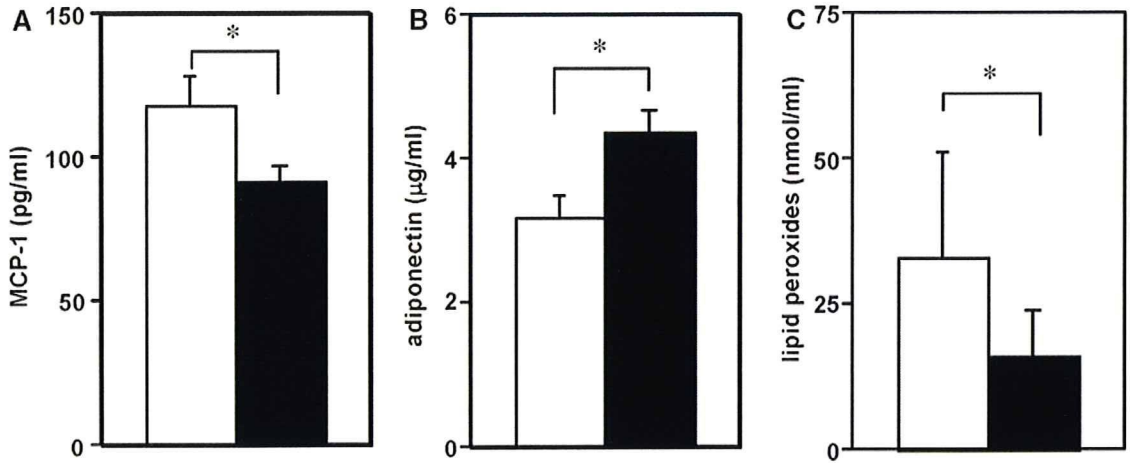

Figure 6. Hepatic LOX-1 expression altered oxidative stress–related plasma parameters. Plasma monocyte chemotactic protein-1 (A), adiponectin (B), and lipid peroxide (C) levels were determined 2 weeks after adenoviral administration to LacZ mice (white bars) and LOX-1 mice (black bars; n=6 per group).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

surface areas of LOX-1 mice. OxLDL reportedly enhances apoptosis of smooth muscle cells.[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[44] and decreases adiponectin expression in adipocytes.[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

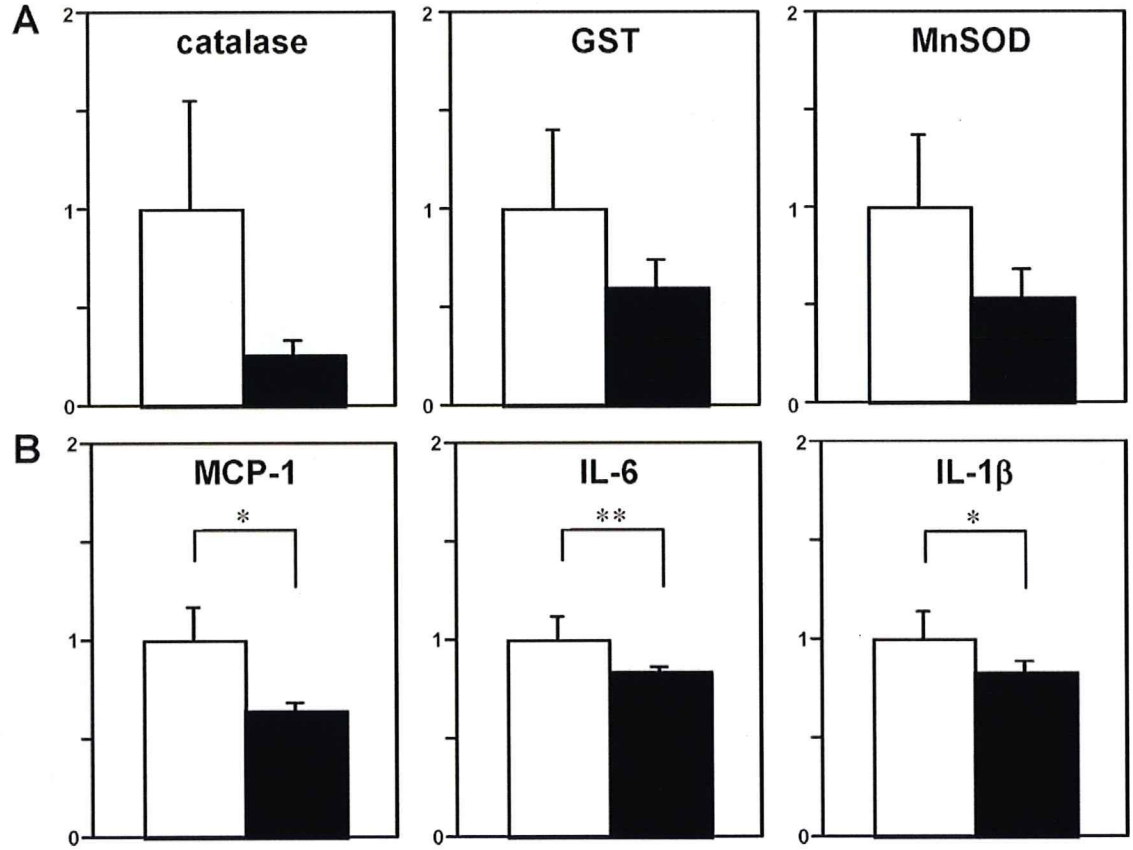

Figure 7. Relative amounts of mRNAs of proteins related to oxidative stress or inflammation in the aorta. On day 5 after LacZ (white bars) or LOX-1 (black bars) adenovirus administration to 24-week-old apoE-deficient mice, relative amounts of mRNA of proteins related to oxidative stress (A) or inflammation (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 dismutase; 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) to Dr Oka from the Ministry of Health, Labor, and Welfare of Japan. This work also was supported by two 21st Century COE Programs (to Drs Katagiri and Oka) from the Ministry of Education, Science, Sports, and Culture.

Disclosures

None.

References

1. Katagiri H, Yamada T, Oka Y. Adiposity and cardiovascular disorders: disturbance of the regulatory system consisting of humoral and neuronal signals. *Circ Res.* 2007;101:27–39.
2. Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. *Science.* 1986;232:34–47.
3. Shepherd J, Cobbe SM, Ford I, Isles CG, Lorimer AR, MacFarlane PW, McKillop JH, Packard CJ. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia: West of Scotland Coronary Prevention Study Group. *N Engl J Med.* 1995;333:1301–1307.
4. Ross R. Atherosclerosis: an inflammatory disease. *N Engl J Med.* 1999; 340:115–126.
5. Yla-Herttuala S, Palinski W, Rosenfeld ME, Parthasarathy S, Carew TE, Butler S, Witztum JL, Steinberg D. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J Clin Invest.* 1989;84:1086–1095.
6. Witztum JL, Steinberg D. Role of oxidized low density lipoprotein in atherogenesis. *J Clin Invest.* 1991;88:1785–1792.
7. Yui S, Sasaki T, Miyazaki A, Horiuchi S, Yamazaki M. Induction of murine macrophage growth by modified LDLs. *Arterioscler Thromb.* 1993;13:331–337.
8. Kugiyama K, Kerns SA, Morrisett JD, Roberts R, Henry PD. Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. *Nature.* 1990;344:160–162.
9. Galle J, Hansen-Hagge T, Wanner C, Seibold S. Impact of oxidized low density lipoprotein on vascular cells. *Atherosclerosis.* 2006;185:219–226.
10. Meagher E, Rader DJ. Antioxidant therapy and atherosclerosis: animal and human studies. *Trends Cardiovasc Med.* 2001;11:162–165.
11. Rimm EB, Stampfer MJ. Antioxidants for vascular disease. *Med Clin North Am.* 2000;84:239–249.
12. Pratico D, Tangirala RK, Rader DJ, Rokach J, FitzGerald GA. Vitamin E suppresses isoprostane generation in vivo and reduces atherosclerosis in ApoE-deficient mice. *Nat Med.* 1998;4:1189–1192.
13. Terasawa Y, Ladha Z, Leonard SW, Morrow JD, Newland D, Sanan D, Packer L, Traber MG, Farese RV Jr. Increased atherosclerosis in hyperlipidemic mice deficient in alpha-tocopherol transfer protein and vitamin E. *Proc Natl Acad Sci U S A.* 2000;97:13830–13834.
14. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial: Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. *Lancet.* 1999;354:447–455.
15. Miller ER 3rd, Pastor-Barriuso R, Dalal D, Riemersma RA, Appel LJ, Guallar E. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. *Ann Intern Med.* 2005;142:37–46.
16. Kodama T, Freeman M, Rohrer L, Zabrecky J, Matsudaira P, Krieger M. Type I macrophage scavenger receptor contains alpha-helical and collagen-like coiled coils. *Nature.* 1990;343:531–535.
17. Endemann G, Stanton LW, Madden KS, Bryant CM, White RT, Protter AA. CD36 is a receptor for oxidized low density lipoprotein. *J Biol Chem.* 1993;268:11811–11816.
18. Krieger M, Acton S, Ashkenas J, Pearson A, Penman M, Resnick D. Molecular flypaper, host defense, and atherosclerosis: structure, binding properties, and functions of macrophage scavenger receptors. *J Biol Chem.* 1993;268:4569–4572.
19. Acton SL, Scherer PE, Lodish HF, Krieger M. Expression cloning of SR-BI, a CD36-related class B scavenger receptor. *J Biol Chem.* 1994; 269:21003–21009.
20. Ramprasad MP, Terpstra V, Kondratenko N, Quehenberger O, Steinberg D. Cell surface expression of mouse macrosialin and human CD68 and their role as macrophage receptors for oxidized low density lipoprotein. *Proc Natl Acad Sci U S A.* 1996;93:14833–14838.
21. Sawamura T, Kume N, Aoyama T, Moriwaki H, Hoshikawa H, Aiba Y, Tanaka T, Miwa S, Katsura Y, Kita T, Masaki T. An endothelial receptor for oxidized low-density lipoprotein. *Nature.* 1997;386:73–77.
22. Kume N, Murase T, Moriwaki H, Aoyama T, Sawamura T, Masaki T, Kita T. Inducible expression of lectin-like oxidized LDL receptor-1 in vascular endothelial cells. *Circ Res.* 1998;83:322–327.
23. Mizuguchi H, Kay MA. Efficient construction of a recombinant adenovirus vector by an improved in vitro ligation method. *Hum Gene Ther.* 1998;9:2577–2583.
24. Mizuguchi H, Kay MA. A simple method for constructing E1- and E1/E4-deleted recombinant adenoviral vectors. *Hum Gene Ther.* 1999; 10:2013–2017.
25. Katagiri H, Asano T, Ishihara H, Inukai K, Shibasaki Y, Kikuchi M, Yazaki Y, Oka Y. Overexpression of catalytic subunit p110alpha of phosphatidylinositol 3-kinase increases glucose transport activity with translocation of glucose transporters in 3T3-L1 adipocytes. *J Biol Chem.* 1996;271:16987–16990.
26. Zhang SH, Reddick RL, Piedrahita JA, Maeda N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science.* 1992;258:468–471.

27. Ishigaki Y, Katagiri H, Yamada T, Ogihara T, Imai J, Uno K, Hasegawa Y, Gao J, Ishihara H, Shimosegawa T, Sakoda H, Asano T, Oka Y. Dissipating excess energy stored in the liver is a potential treatment strategy for diabetes associated with obesity. *Diabetes*. 2005;54:322–332.
28. Gao J, Katagiri H, Ishigaki Y, Yamada T, Ogihara T, Imai J, Uno K, Hasegawa Y, Kanzaki M, Yamamoto TT, Ishibashi S, Oka Y. Involvement of apolipoprotein E in excess fat accumulation and insulin resistance. *Diabetes*. 2007;56:24–33.
29. Innerarity TL, Pitas RE, Mahley RW. Lipoprotein-receptor interactions. *Methods Enzymol*. 1986;129:542–565.
30. Devaraj S, Hugou I, Jialal I. Alpha-tocopherol decreases CD36 expression in human monocyte-derived macrophages. *J Lipid Res*. 2001; 42:521–527.
31. Fujino T, Asaba H, Kang MJ, Ikeda Y, Sone H, Takada S, Kim DH, Ioka RX, Ono M, Tomoyori H, Okubo M, Murase T, Kamataki A, Yamamoto J, Magoori K, Takahashi S, Miyamoto Y, Oishi H, Nose M, Okazaki M, Usui S, Imaizumi K, Yanagisawa M, Sakai J, Yamamoto TT. Low-density lipoprotein receptor-related protein 5 (LRP5) is essential for normal cholesterol metabolism and glucose-induced insulin secretion. *Proc Natl Acad Sci U S A*. 2003;100:229–234.
32. Yamada T, Katagiri H, Ishigaki Y, Ogihara T, Imai J, Uno K, Hasegawa Y, Gao J, Ishihara H, Niijima A, Mano H, Aburatani H, Asano T, Oka Y. Signals from intra-abdominal fat modulate insulin and leptin sensitivity through different mechanisms: neuronal involvement in food-intake regulation. *Cell Metab*. 2006;3:223–229.
33. Reddick RL, Zhang SH, Maeda N. Atherosclerosis in mice lacking apo E: evaluation of lesional development and progression. *Arterioscler Thromb*. 1994;14:141–147.
34. Uno K, Katagiri H, Yamada T, Ishigaki Y, Ogihara T, Imai J, Hasegawa Y, Gao J, Kaneko K, Iwasaki H, Ishihara H, Sasano H, Inukai K, Mizuguchi H, Asano T, Shiota M, Nakazato M, Oka Y. Neuronal pathway from the liver modulates energy expenditure and systemic insulin sensitivity. *Science*. 2006;312:1656–1659.
35. Okamoto Y, Kihara S, Ouchi N, Nishida M, Arita Y, Kumada M, Ohashi K, Sakai N, Shimomura I, Kobayashi H, Terasaka N, Inaba T, Funahashi T, Matsuzawa Y. Adiponectin reduces atherosclerosis in apolipoprotein E–deficient mice. *Circulation*. 2002;106:2767–2770.
36. Cushing SD, Berliner JA, Valente AJ, Territo MC, Navab M, Parhami F, Gerrity R, Schwartz CJ, Fogelman AM. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc Natl Acad Sci U S A*. 1990;87: 5134–5138.
37. Soares AF, Guichardant M, Cozzone D, Bernoud-Hubac N, Bouzaidi-Tiali N, Lagarde M, Geloen A. Effects of oxidative stress on adiponectin secretion and lactate production in 3T3-L1 adipocytes. *Free Radic Biol Med*. 2005;38:882–889.
38. Nissen SE, Tuzcu EM, Schoenhagen P, Brown BG, Ganz P, Vogel RA, Crowe T, Howard G, Cooper CJ, Brodie B, Grines CL, DeMaria AN. Effect of intensive compared with moderate lipid-lowering therapy on progression of coronary atherosclerosis: a randomized controlled trial. *JAMA*. 2004;291:1071–1080.
39. Yonemura A, Momiyama Y, Fayad ZA, Ayaori M, Ohmori R, Higashi K, Kihara T, Sawada S, Iwamoto N, Ogura M, Taniguchi H, Kusuhara M, Nagata M, Nakamura H, Tamai S, Ohsuzu F. Effect of lipid-lowering therapy with atorvastatin on atherosclerotic aortic plaques detected by noninvasive magnetic resonance imaging. *J Am Coll Cardiol*. 2005;45: 733–742.
40. Matsuzaki M, Hiramori K, Imaizumi T, Kitabatake A, Hishida H, Nomura M, Fujii T, Sakuma I, Fukami K, Honda T, Ogawa H, Yamagishi M. Intravascular ultrasound evaluation of coronary plaque regression by low density lipoprotein-apheresis in familial hypercholesterolemia: the Low Density Lipoprotein-Apheresis Coronary Morphology and Reserve Trial (LACMART). *J Am Coll Cardiol*. 2002;40:220–227.
41. Jovinge S, Crisby M, Thyberg J, Nilsson J. DNA fragmentation and ultrastructural changes of degenerating cells in atherosclerotic lesions and smooth muscle cells exposed to oxidized LDL in vitro. *Arterioscler Thromb Vasc Biol*. 1997;17:2225–2231.
42. Nishio E, Arimura S, Watanabe Y. Oxidized LDL induces apoptosis in cultured smooth muscle cells: a possible role for 7-ketocholesterol. *Biochem Biophys Res Commun*. 1996;223:413–418.
43. Okura Y, Brink M, Itabe H, Scheidegger KJ, Kalangos A, Delafontaine P. Oxidized low-density lipoprotein is associated with apoptosis of vascular smooth muscle cells in human atherosclerotic plaques. *Circulation*. 2000; 102:2680–2686.
44. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest*. 2004;114:1752–1761.
45. Meagher EA, Barry OP, Lawson JA, Rokach J, FitzGerald GA. Effects of vitamin E on lipid peroxidation in healthy persons. *JAMA*. 2001;285: 1178–1182.
46. Bowry VW, Ingold KU, Stocker R. Vitamin E in human low-density lipoprotein: when and how this antioxidant becomes a pro-oxidant. *Biochem J*. 1992;288(pt 2):341–344.

CLINICAL PERSPECTIVE

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, oxLDL plays very important roles in atherosclerosis formation, and the underlying mechanisms may involve both direct (foam cell formation) and indirect (increased oxidative stress) effects. In addition, smooth muscle cells in the surface areas of atherosclerotic plaques were increased in LOX-1 mice, suggesting that oxLDL makes plaques vulnerable, possibly leading to plaque ruptures. 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.

Table

Sequences of Quantitative RT-PCR primers

Probe	Primer 1	Primer 2
MCP-1	5'-actgaagccagctctctcttcctc-3'	5'-ttccttcttggggtcagcacagac-3'
IL-6	5'-caatgctctcctaacagataag-3'	5'-aggcataacgcactaggt-3'
IL-1β	5'-caagcaatacccaaagaaga-3'	5'-gaaacagtccagcccatac-3'
CRP	5'-cagcttctctcggacttttg-3'	5'-aggtgttcagtggcttctttg-3'
TNFα	5'-aggtcaatctgcccaagt-3'	5'-gggctgggtagagaatg-3'
catalase	5'-aggtgttgaacgaggagga-3'	5'-ctcagcgttgtacttgtcca-3'
GST	5'-tgccaagatcaaggaacaaa-3'	5'-ccacatggtagaggagttcaa-3'
MnSOD	5'-ggtcgcttacagattgct-3'	5'-ctcccagttgattacattcc-3'
GAPDH	5'- accacagtccatgccatcac -3'	5'- tccaccaccctgttgctgta -3'

Cardiovascular Research (2008) 79, 287–293
doi:10.1093/cvr/cvn110

EUROPEAN SOCIETY OF CARDIOLOGY®

LOX-1 deletion decreases collagen accumulation in atherosclerotic plaque in low-density lipoprotein receptor knockout mice fed a high-cholesterol diet

Changping Hu[1,2†], Abhijit Dandapat[1†], Liuqin Sun[1,3], Jiawei Chen[1], Muhammad R. Marwali[1], Francesco Romeo[4], Tatsuya Sawamura[5], and Jawahar L. Mehta[1*]

[1]*Department of Internal Medicine, Central Arkansas Veterans Healthcare System and University of Arkansas for Medical Sciences, 4301 West Markham Street, Slot 532, Little Rock, AR 72205, USA;* [2]*Department of Pharmacology, School of Pharmaceutical Sciences, Central South University, Changsha, China;* [3]*Department of Ophthalmology, Heping Hospital, Changzhi Medical College, Changzhi, China;* [4]*Department of Cardiology, University of Rome 'Tor Vergata', Rome, Italy; and* [5]*Department of Vascular Physiology, National Cardiovascular Center Research Institute, Suita, Osaka, Japan*

Received 13 January 2008; revised 22 April 2008; accepted 25 April 2008; online publish-ahead-of-print 3 May 2008

Time for primary review: 21 days

KEYWORDS
Atherosclerosis;
LOX-1;
Extracellular matrix;
Matrix metalloproteinases;
NADPH oxidase

Aims Collagen, as a component of the extracellular matrix, has been linked to atherosclerotic plaque formation and stability. Activation of LOX-1, a lectin-like oxidized low-density lipoprotein (LDL) receptor-1, exerts a significant role in collagen formation. We examine the hypothesis that LOX-1 deletion may inhibit collagen accumulation in atherosclerotic arteries in LDL receptor (LDLR) knockout (KO) mice.

Methods and results We generated LOX-1 KO and LOX-1/LDLR double KO mice on a C57BL/6 (wild-type mice) background and fed a 4% cholesterol/10% cocoa butter diet for 18 weeks. Vessel wall collagen accumulation was increased in association with atherogenesis in the LDLR KO mice ($P < 0.01$ vs. wild-type mice), but much less so in the double KO mice ($P < 0.01$ vs. LDLR KO mice). Collagen accumulation data were corroborated with pro-collagen I measurements. Expression/activity of osteopontin, fibronectin, and matrix metalloproteinases (MMP-2 and MMP-9) was also increased in the LDLR KO mice ($P < 0.01$ vs. wild-type mice), but not in the mice with LOX-1 deletion ($P < 0.01$ vs. LDLR KO mice). The expression of NADPH oxidase ($p47^{phox}$, $p22^{phox}$, $gp91^{phox}$, and Nox-4 subunits) and nitrotyrosine was increased in the LDLR KO mice ($P < 0.01$ vs. wild-type mice) and not in mice with LOX-1 deletion ($P < 0.01$ vs. LDLR KO mice). Phosphorylation of Akt-1 and endothelial nitric oxide synthase and expression of haem-oxygenase-1 were found to be reduced in the LDLR KO mice ($P < 0.01$ vs. wild-type mice), but not in the mice with LOX-1 deletion ($P < 0.01$ vs. LDLR KO mice).

Conclusion LOX-1 deletion reduces enhanced collagen deposition and MMP expression in atherosclerotic regions via inhibition of pro-oxidant signals.

1. Introduction

Atherogenesis involves lipid accumulation, especially oxidized low-density lipoproteins (ox-LDLs),[1] endothelial injury,[2] chronic inflammation[3] and oxidative stress.[4] Besides the accumulation of lipids, atherosclerotic regions are characterized by the presence of fibrous elements in different layers of the arterial wall. A new insight into the role of collagen accumulation in plaque development has been gained by understanding the role of extracellular matrix (ECM).[5] ECM composition and its metabolic behaviour are decisive factors in the evolution and complications of atherosclerosis. It is evident that the metabolic balance of ECM is regulated in large part by matrix metalloproteinases (MMPs).[5]

LOX-1, a lectin-like receptor for ox-LDL, is a major receptor responsible for binding, internalization, and degradation of ox-LDL in endothelial cells.[6] It has been demonstrated that insertion of LOX-1 plasmids in cardiac fibroblasts that are naturally low expressors of LOX-1 alters the biology of fibroblasts to pro-inflammatory phenotype.[7] Further, ox-LDL treatment enhances collagen formation by fibroblasts that can be blocked by anti-LOX-1 antibody. In cultured cardiac myocytes, LOX-1 activation has also been shown to upregulate the expression of pro-collagen I and collagen type I via redox-sensitive pathways.[8] A recent study shows that LOX-1 deletion alters signals of myocardial

† These authors contributed equally to this work.
* Corresponding author. Tel: +1 501 686 5046; fax: +1 501 686 6180.
E-mail address: mehtaJL@uams.edu

remodelling immediately after ischaemia.[9] These observations collectively suggest that LOX-1 may be an important player in the regulation of ECM.

There is growing evidence to support the contributory role of LOX-1 in atherogenesis.[6] Importantly, targeted deletion of LOX-1 reduces atherogenesis in the LDL receptor knockout (LDLR KO) mice fed high-cholesterol diet.[10] In order to gain further insight into the role of LOX-1 in ECM formation in atherogenesis, we studied the effect of LOX-1 deletion on ECM accumulation and MMPs expression in atherosclerotic aortas. We also examined the modulation of oxidative stress and pro-inflammatory signals as the mechanistic basis of ECM modulation.

2. Methods

2.1 Animal protocol

The generation of LOX-1 KO and LOX-1/LDLR double KO mice has been described recently.[10] In brief, C57BL/6 mice (also referred to as wild-type mice) and homozygous LDLR KO mice (on C57BL/6 background) were originally obtained from Jackson Laboratories. The homozygous LOX-1 KO and LOX-1/LDLR double KO mice were backcrossed eight times with C57BL/6 strain to replace the genetic background. C57BL/6 and homozygous LOX-1 KO, LDLR KO and LDLR/LOX-1 double KO (all on C57BL/6 background) mice were bred by brother–sister mating and housed in the breeding colony at University of Arkansas for Medical Sciences, Little Rock, AR, USA. All male animals were given a high-cholesterol diet (4% cholesterol/10% cocoa butter) for 18 weeks from the age of 6 weeks. This investigation conforms to the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health. All experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Usage Committee.

2.2 Quantitative analysis of collagen-positive area

Entire aortas from the aortic arch above the aortic valves to the iliac bifurcation were harvested and embedded in paraffin. Cross-sections (5 μm thick) were made at five pre-defined points (proximal ascending aorta, aortic arch, descending aorta, mid-thoracic aorta, and abdominal aorta above the renal arteries).[10] The sections were stained with Masson's trichrome and Picro-sirius red. The images were captured by digital imaging system and analysed with Image pro software (Media Cybernetics). The presence of area positive for collagen (blue) was recorded for each section, averaged for each mouse and expressed as ratio of entire vessel wall area. Data were obtained from five mice in each group.

2.3 Immunohistochemical staining

Sections (5 μm thick) of aortas made at five pre-defined points as described above were incubated with primary antibody to fibronectin, osteopontin, or nitrotyrosine (Santa Cruz, dilution 1:200) for 2 h at room temperature, rinsed with PBS, and incubated with corresponding biotinylated secondary antibody for 30 min. The slides were then incubated in avidin–biotin complex for 30 min followed by rinse with PBS, then incubated in diaminobenzidine, and finally washed in distilled water and counterstained with hematoxylin.

2.4 Protein preparation and analysis by western blot

Aortic specimens were derived from animals at 18 weeks of high-cholesterol diet. Aortic protein was extracted for expression analysis of pro-collagen I, osteopontin, fibronectin, MMP-2, MMP-9, NADPH oxidase $p22^{phox}$, NADPH oxidase $p47^{phox}$, NADPH oxidase $gp91^{phox}$, NADPH oxidase Nox-4, nitrotyrosine, Akt-1, phos-Akt-1 (Ser 473), phos-S1177 endothelial nitric oxide synthase (eNOS), haem-oxygenase-1 (HO-1), and β-actin using standard methodologies of western blot.[8] Band density relative to β-actin was analysed.

2.5 MMP-2 and MMP-9 activity assay

Activity of MMP-2 and MMP-9 in aortic tissues was determined by zymography.[8]

2.6 Statistical analysis

Data are presented as mean ± SE. All data were analysed by a two-way ANOVA with a Bonferroni *post hoc* test. Four-fold comparisons were performed and an adjusted value of $P < 0.05$ was considered to be significant. All calculations were performed with SPSS version 12.0.

3. Results

3.1 LOX-1 deletion reduces collagen deposition in the aortic wall

Collagen is an important component of atherosclerotic plaque, and its role as a component of ECM has been established.[5] We, therefore, determined accumulation of collagen by specific staining in multiple aortic sections from different animal groups. The results of Masson's trichrome and Picro-sirius red staining were similar. Representative examples of aortic sections are shown in *Figure 1A*, and the summary data from Masson's trichrome staining are shown in *Figure 2A*. There was extensive deposition of collagen in the aortas of LDLR KO mice, encompassing ~40% of the aortic cross-sectional area. In comparison to the LDLR KO mice, collagen deposition was much less in the aorta of double KO mice (~50% reduction vs. LDLR KO mice, $P < 0.01$). The wild-type control mice also showed some collagen-positive areas (~18% of cross-sectional area), perhaps a result of high-cholesterol diet feeding, and that there were much fewer collagen-positive areas in the LOX-1 KO mice.

In support of the collagen accumulation data, the LDLR KO mice showed a marked increase in pro-collagen I expression ($P < 0.01$ vs. wild-type mice). Deletion of LOX-1 in the LDLR KO mice reduced pro-collagen I expression ($P < 0.01$ vs. LDLR KO mice). Importantly, the pro-collagen I expression was lower in the LOX-1 KO mice when compared with that in the wild-type mice ($P < 0.01$, *Figure 2B*), indicating that LOX-1 deletion also reduces the basal expression of pro-collagen I.

3.2 LOX-1 deletion reduces expression of osteopontin and fibronectin in the aortic wall

Osteopontin has been shown to interact with fibronectin and to play an important role in ECM organization and stability.[11] Accordingly, we determined the expression of osteopontin and fibronectin in the aortic sections from different groups of mice. Representative examples (immunohistochemistry) are shown in *Figures 1B* and western analysis in *Figure 3*. The expression of osteopontin as well as fibronectin was increased in the LDLR KO mice ($P < 0.01$ vs. wild-type mice). In contrast, LDLR/LOX-1 double KO mice showed much less increase in the expression of osteopontin as well as fibronectin ($P < 0.01$ vs. LDLR KO mice).

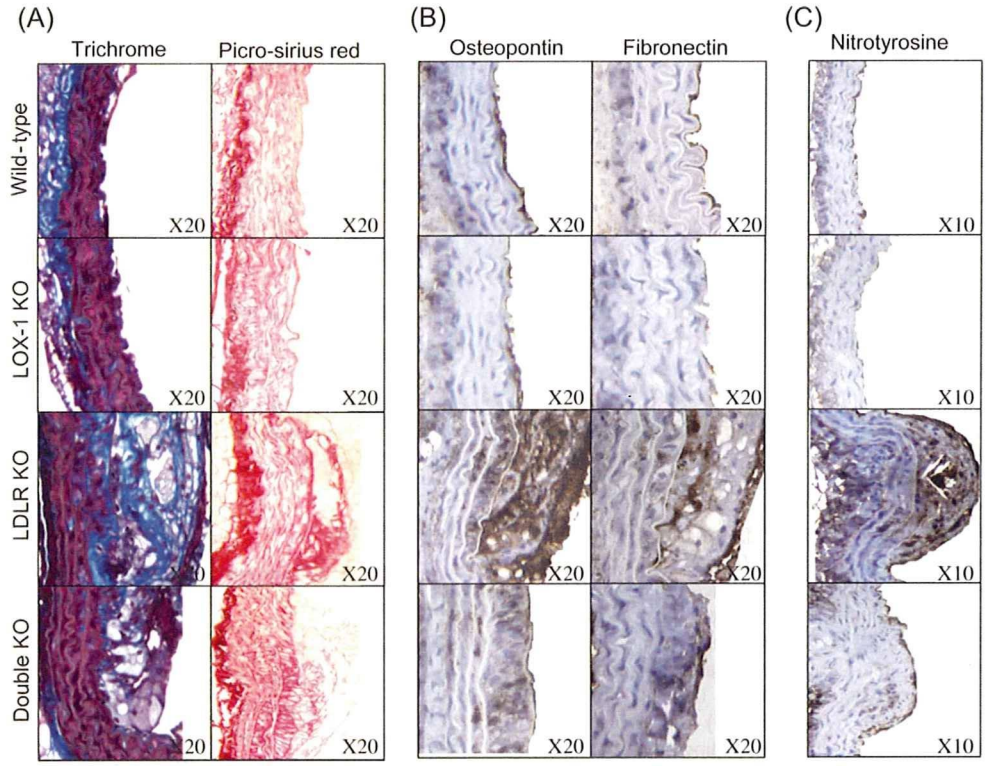

Figure 1 Representative examples of collagen accumulation (Picro-sirius and Masson's trichrome staining, *A*), osteopontin and fibronectin expression, *B*, and nitrotyrosine staining, *C*) in selected aortic sections.

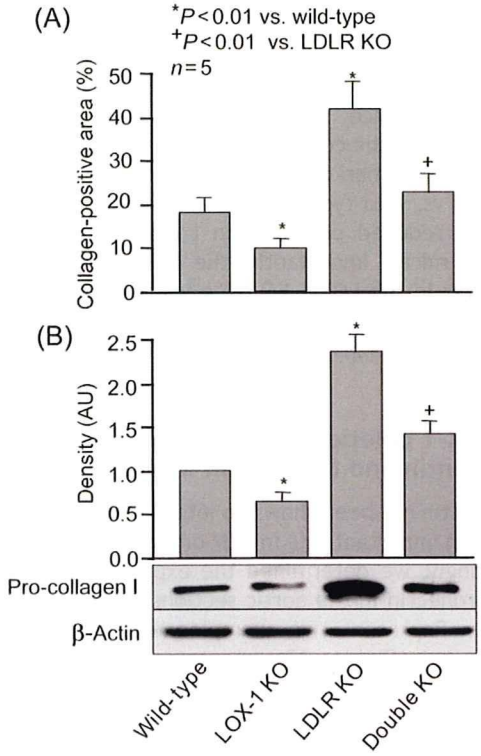

Figure 2 Quantitation of collagen accumulation (*A*) and expression of pro-collagen I by western blot analysis (*B*). KO, knockout.

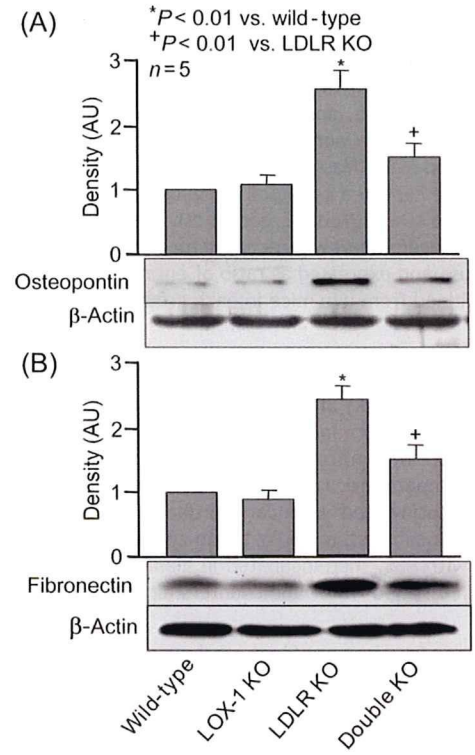

Figure 3 Representative examples and quantitation of expression of osteopontin (*A*) and fibronectin (*B*), determined by western blot analysis.

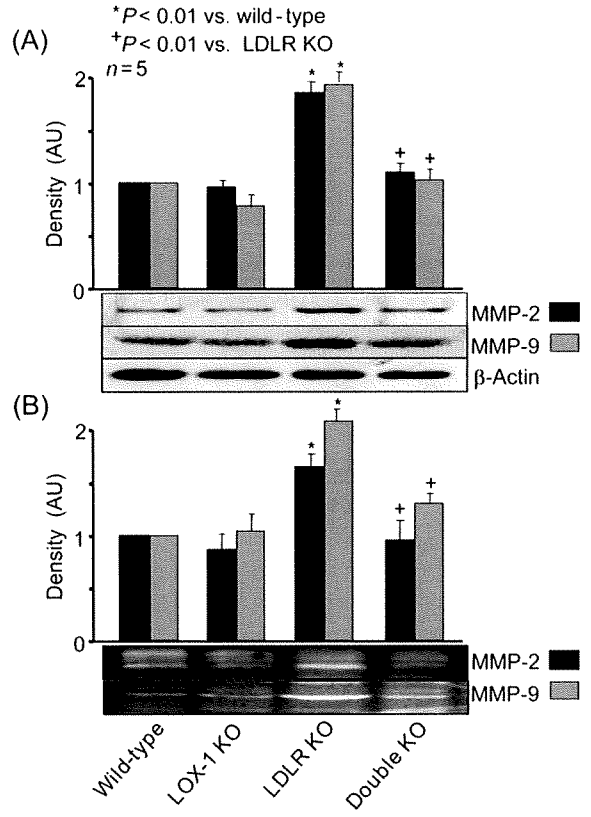

Figure 4 Representative examples and quantitation of expression of matrix metalloproteinases (MMPs), determined by western blot analysis (*A*) and of MMP activity, determined by zymography (*B*).

3.3 LOX-1 deletion reduces matrix metalloproteinases expression and activity in the aortic wall

Collagen accumulation in various tissues depends not only on its production, but also on its degradation by proteinases, such as MMPs.[12] Therefore, we determined the expression and activity of mouse-specific MMP-2 and MMP-9 (*Figure 4A* and *B*). The expression as well as activity of MMP-2 and MMP-9 was found to be increased ≈100% in LDLR KO ($P <$ 0.01 vs. wild-type mice). On the other hand, deletion of LOX-1 in the LDLR KO mice 'normalized' the expression and activity of MMPs ($P < 0.01$ vs. LDLR KO mice; $P > 0.05$ vs. wild-type mice).

3.4 LOX-1 deletion reduces redox-sensitive signalling associated with collagen accumulation

Atherosclerosis involves oxidative stress and inflammation.[13,14] In keeping with this concept, $p47^{phox}$, $p22^{phox}$, $gp91^{phox}$, and Nox-4 subunits of NADPH oxidase were markedly increased in the LDLR KO mice ($P < 0.01$ vs. wild-type mice) (*Figure 5A*). The upregulation of four subunits of NADPH oxidase was reduced by LOX-1 deletion in the LDLR KO mice. It is noteworthy that the expression of all four subunits of NADPH oxidase was lower in the LOX-1 KO mice ($P <$ 0.01 vs. wild-type mice), indicating that LOX-1 deletion reduces the basal expression of four subunits of NADPH oxidase. To confirm the increased production of reactive oxidative species (ROS) in aorta, we examined the presence of nitrotyrosine as an indirect marker of oxidative stress by immunohistochemistry (*Figure 1C*) and western blot analysis

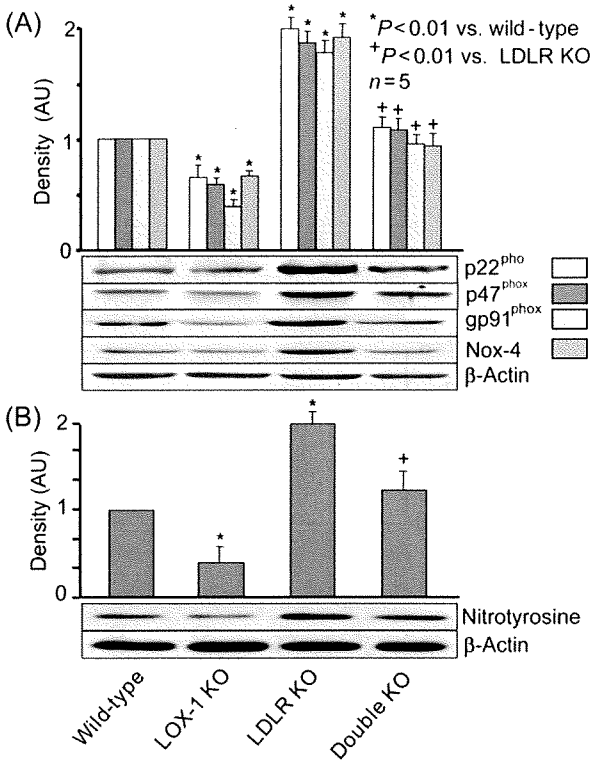

Figure 5 Representative examples and quantitation of the expression of NADPH oxidases ($p47^{phox}$, $p22^{phox}$, $gp91^{phox}$, and Nox-4 subunits) (*A*) and nitrotyrosine (*B*), determined by western blot analysis.

(*Figure 5B*). Expression of nitrotyrosine was higher in LDLR KO mice ($P < 0.01$ vs. the wild-type mice), and LOX-1 deletion limited this increase in nitrotyrosine expression.

Next, we studied the expression of Akt-1 protein and its phosphorylation in the aortic tissues. While Akt-1 protein expression was similar in all mice, Akt-1 phosphorylation was reduced by 50% in the LDLR KO mice ($P < 0.01$ vs. wild-type mice), and LOX-1 deletion in the wild-type and LDLR KO mice 'normalized' Akt-1 phosphorylation (*Figure 6A*).

Akt-1 activation regulates the activity of eNOS. We, therefore, examined phos-S1177 eNOS expression in the aortic tissues. As shown in *Figure 6B*, the expression of phos-S1177 eNOS was reduced by ~50% in the LDLR KO mice compared with the wild-type mice ($P < 0.01$), and LOX-1 deletion markedly increased eNOS phosphorylation in the LDLR mice ($P < 0.01$). It is of note that the basal level of eNOS activity was higher in the LOX-1 KO mice when compared with wild-type mice ($P < 0.01$).

We also examined the expression of HO-1, another vasodilator species that is relevant in atherogenesis.[15,16] As shown in *Figure 6C*, HO-1 expression was reduced in the aortas from LDLR KO mice (vs. wild-type mice). On the other hand, LOX-1 deletion enhanced HO-1 expression in LDLR KO mice. As with eNOS activity, basal levels of HO-1 were higher in the LOX-1 KO mice (vs. wild-type mice, $P < 0.01$).

4. Discussion

Atherosclerosis has been viewed as uncontrolled plaque growth eventually leading to total occlusion of the artery. New clinical findings suggest that acute coronary events are triggered by thrombosis associated with rupture of the

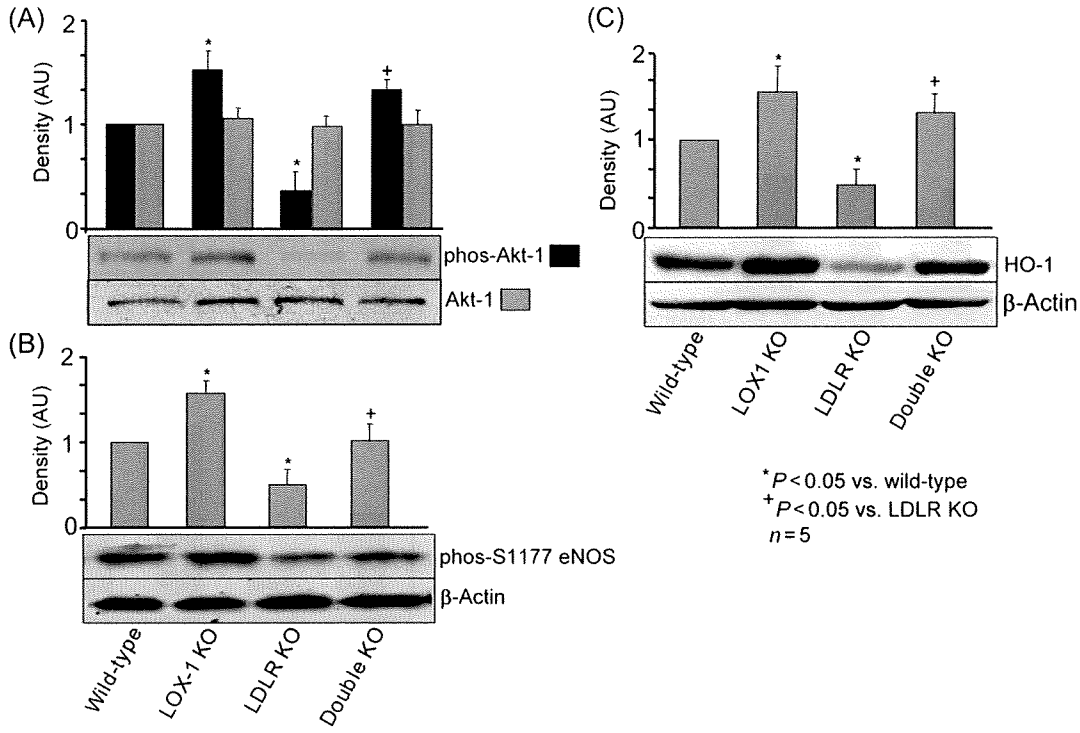

Figure 6 Representative examples and quantitation of the expression of Akt-1 (*A*), phos-S1177 eNOS (*B*), and HO-1 (*C*), determined by western blot analysis. eNOS, endothelial nitric oxide synthase; HO-1, haem-oxygenase-1; phos, phosphorylated.

atherosclerotic plaque.[17] Clinical data from analysis of human atherosclerotic regions by intravascular ultrasound histology and polarization-sensitive optical coherence tomography suggest that collagen deposition (fibrous tissue) is a significant component of the atherosclerotic region.[18,19] Detailed analysis of the atherosclerotic plaques in animal tissues also shows that collagen constitutes over 50% of the plaques.[20] It may be postulated that fibrous tissue provides an anchor for smooth muscle cells and monocytes/macrophages. In light of our previous *in vitro* studies[7] showing that collagen formation by fibroblasts treated with ox-LDL is an oxidant-response that can be blocked by LOX-1 abrogation, we embarked on this *in vivo* study to examine if LOX-1 deletion in the LDLR KO mice will attenuate collagen deposition. Indeed, our study shows that collagen deposition is markedly reduced in the LDLR KO mice with LOX-1 deletion. Further, this study revealed that the signals involved in collagen deposition in atherosclerotic tissues are similar to those seen in *in vitro* studies.

4.1 Collagen formation and deposition

While almost all cell types in atherosclerotic regions form collagen, fibroblasts are the primary source of collagen. Our previous studies showed that treatment of fibroblasts with ox-LDL enhances collagen formation, especially when LOX-1 is over-expressed in fibroblasts.[7] Treatment of fibroblasts with angiotensin II (Ang II), which is abundantly present in the atherosclerotic regions,[21] also stimulates fibroblast growth and collagen formation.[22] In addition to collagen formation, there is abundance of collagen degrading enzymes, the MMPs, in the atherosclerotic plaque.[5] Collagen degradation products and MMPs can also be identified in plasma and urine of patients with atherosclerosis.[23] However, these markers lack a specific 'tissue signature'.[20]

Simultaneous increase in MMPs and collagen expression in atherosclerotic tissues suggests that the two processes are inter-related and represent a cellular attempt to regulate the remodelling process. The expression of both MMPs and collagen may be a response to ROS release, a common accompaniment of atherogenesis.[13] We have earlier shown that atherosclerosis is associated with over-expression of LOX-1, a finding reproduced in the LDLR KO mice on high-fat diet.[10] It is of note that LOX-1 activation has been linked to ox-LDL, Ang II, and release of ROS.[6]

We showed that LOX-1 deletion reduced the extent of atherosclerosis in the LDLR KO mice,[10] and also attenuated the expression/activity of MMPs and pro-collagen I. Reduction in pro-collagen I may represent a decrease in oxidant stress in the LOX-1 KO mice. NADPH oxidase is the major source of ROS in the vascular tissues.[24] We measured the expression of NADPH oxidase and nitrotyrosine, and found that the expression of NADPH oxidase ($p22^{phox}$, $p47^{phox}$, $pg91^{phox}$, and Nox-4 subunits) and nitrotyrosine was increased dramatically in the LDLR KO mice. This increase in NADPH oxidase and nitrotyrosine (oxidant stress marker) was much less in mice with LOX-1 deletion.

We do not know the exact source of NADPH oxidase and ROS in the atherosclerotic arteries, but it could be fibroblasts, smooth muscle cells, endothelial cell, and/or inflammatory cells. All these cell types have been shown to generate ROS;[24] it also appeared to be confirmed by nitrotyrosine staining in our study (*Figure 1C*).

4.2 Osteopontin and fibronectin expression in atherosclerosis

We observed that the expression of osteopontin as well as fibronectin was increased in the aortas of LDLR KO mice compared with wild-type mice. Osteopontin has been

implicated in chemoattraction of monocytes and in cell-mediated immunity.[25] It is also important in smooth muscle cell migration.[26] Collins *et al.*[27] showed that osteopontin was formed in response to Ang II, and the osteopontin null mice had much less fibroblasts proliferation and much less ECM accumulation after 3 weeks of Ang II infusion. Interestingly, osteopontin null mice also had reduced atherosclerosis and MMPs activity.[28]

The signal for osteopontin expression seems to involve oxidant stress and related pathways. Xie *et al.*[29] showed that Ang II regulates osteopontin gene expression via ROS-sensitive signalling pathway. Lai *et al.*[30] demonstrated that osteopontin, via activation of NADPH oxidase-derived superoxide anion formation, promotes upregulation of MMP-9 in primary aortic myofibroblasts and smooth muscle cells under hyperglycaemic conditions *in vitro*. Gorin *et al.*[31] have similarly shown a relationship between NADPH oxidase activation and fibronectin generation in both *in vitro* and *in vivo* conditions. Our previous study[10] showed that p38MAPK activity is increased in the LDLR KO mice and much less so in the LDLR KO mice with LOX-1 deletion. The results of the present study coupled with previous work suggest a strong link between NADPH oxidase-induced oxidant stress, osteopontin/fibronectin expression, and MMPs expression. In keeping with these studies, it was not surprising that the expression of osteopontin, fibronectin, and MMPs was lower in the aortas of mice with LOX-1 abrogation that had low levels of NADPH oxidase ($p22^{phox}$, $p47^{phox}$, $gp91^{phox}$, and Nox-4 subunits).

4.3 Endothelial nitric oxide synthase and HO-1 in atherosclerosis and the effect of LOX-1 deletion

Atherosclerotic regions have reduced activity of eNOS.[32] This phenomenon was confirmed in the present study (*Figure 6*) as well as in our previous work.[10] Reduction of locally released NO may enhance oxidative stress and subsequently monocyte accumulation, collagen synthesis, and cell proliferation. Protein kinase B/Akt-1 is important in downstream targeting of extracellular PI-3 kinase signalling, and alterations in its activity may be important in the phosphorylation of NOS in response to oxidant stimuli.[33] In keeping with this concept, we observed a reduction in Akt-1 phophorylation and diminished expression of eNOS in the aortas of LDLR KO mice. Importantly, LOX-1 deletion enhanced Akt-1 phosphorylation to a level higher than that in the wild-type mice. Previous *in vivo* studies have also documented that LOX-1 is key to altered endothelium-dependent vasorelaxation in atherosclerosis.[10]

There is emerging evidence that HO-1 and its products function as adaptive molecules against oxidative insults.[15,16] HO-1 upregulation in turn reduces NADPH oxidase activity[34] and NF-κB phosphorylation.[35] The signalling of HO-1 in atherogenesis is not clear, but it appears that persistent oxidant stress may reduce the expression of HO-1.[36] There is also evidence that Akt-1 activation upregulates HO-1.[37] The absence of HO-1 exacerbates atherosclerotic lesion formation,[38] suggesting a potential tissue protective role for HO-1 in atherogenesis. In keeping with these observations, we found that atherosclerotic aortas from LDLR KO mice had reduced expression of HO-1. With LOX-1 deletion, there was a marked increase in HO-1 expression. Whether the two events are related or not is not clear, but our observations strongly suggest that oxidant stress and HO-1 are intertwined, and Akt-1/ NF-κB phosphorylation may relate to these alterations.

4.4 Collagen deposition in atherosclerosis and its relevance to LOX-1 deletion

Enhanced expression of collagen appears to be an inherent part of the atherosclerotic process. A host of mediators of oxidative stress, including ox-LDL and Ang II, are present in the atherosclerotic regions and activate NADPH oxidase system. The intense oxidant stress in the atherosclerotic regions stimulates MAPKs and the redox-sensitive transcription factors, such as NF-κB[10] followed by upregulation of genes, such as fibronectin, osteopontin, collagen, and MMPs, which result in the formation of collagen. Interestingly, excessive collagen deposition is associated with enhanced release of MMPs. While ox-LDL and Ang II-stimulated LOX-1 activation enhances oxidative stress and inflammation,[6] and oxidative stress *per se* upregulates LOX-1 expression.[6] This process may self-amplify leading to intense collagen deposition in atherosclerotic regions over time. These events are summarized in *Figure 7*.

Attenuation of the expression of the redox-sensitive signals and collagen formation with LOX-1 deletion suggests that LOX-1 could be a relevant therapeutic target in the management of atherosclerosis and vascular remodelling process.

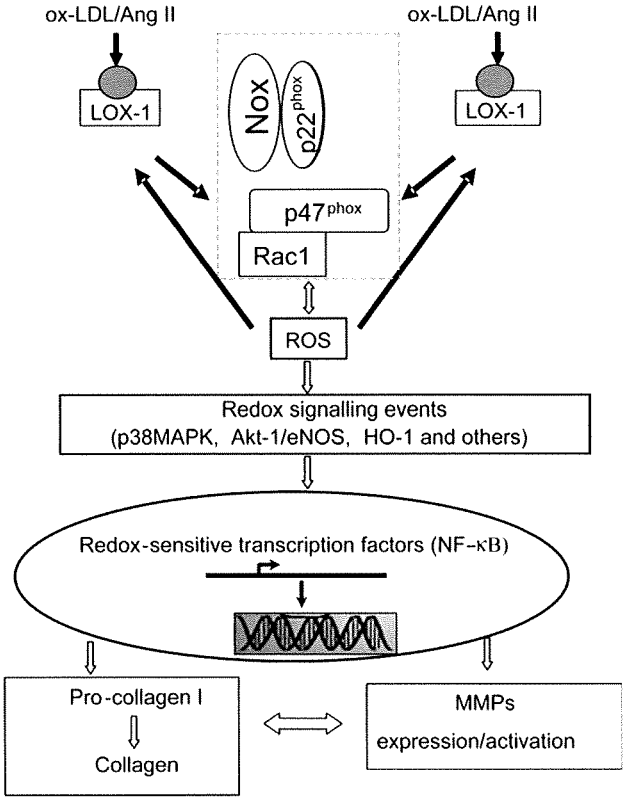

Figure 7 Hypothesized pathways of LOX-1-mediated collagen and MMPs. Mediators of oxidative stress, including oxidized LDL (ox-LDL) and angiotensin II (Ang II), induce LOX-1 expression and resultant activation of NADPH oxidase and reactive oxygen species (ROS) generation. ROS in turn upregulates LOX-1 expression. ROS causes activation of MAPKs followed by transcription of redox-sensitive transcription factor/s which induce the gene for collagen. MMPs are activated as auto-regulatory response to collagen synthesis.