Critical Role of Macrophage 12/15-Lipoxygenase for Atherosclerosis in Apolipoprotein E–Deficient Mice

Yuqing Huo, MD, PhD*; Lei Zhao, MD*; Matthew Craig Hyman, BS; Pavel Shashkin, PhD; Brian L. Harry, BS; Tracy Burcin, MS; S. Bradley Forlow, PhD; Matthew A. Stark, BS; David F. Smith, MS; Sean Clarke, BS; Suseela Srinivasan, PhD; Catherine C. Hedrick, PhD; Domenico Praticò, MD; Joseph L. Witztum, MD; Jerry L. Nadler, MD; Colin D. Funk, PhD; Klaus Ley, MD

Background—Mice lacking leukocyte type 12/15-lipoxygenase (12/15-LO) show reduced atherosclerosis in several models. 12/15-LO is expressed in a variety of cells, including vascular cells, adipocytes, macrophages, and cardiomyocytes. The purpose of this study was to determine which cellular source of 12/15-LO is important for atherosclerosis.

Methods and Results—Bone marrow from 12/15-LO−/−/apoE−/− mice was transplanted into apoE−/− mice and vice versa. Deficiency of 12/15-LO in bone marrow cells protected apoE−/− mice fed a Western diet from atherosclerosis to the same extent as complete absence of 12/15-LO, although plasma 8,12-iso-iPF3α-IV, a measure of lipid peroxidation, remained elevated. 12/15-LO−/−/apoE−/− mice regained the severity of atherosclerotic lesion typical of apoE−/− mice after replacement of their bone marrow cells with bone marrow from apoE−/− mice. Peritoneal macrophages obtained from wild-type but not 12/15-LO−/− mice caused endothelial activation in the presence of native LDL. Absence of 12/15-LO decreased the ability of macrophages to form foam cells when exposed to LDL.

Conclusions—We conclude that macrophage 12/15-LO plays a dominant role in the development of atherosclerosis by promoting endothelial inflammation and foam cell formation. (Circulation. 2004;110:2024-2031.)

Key Words: atherosclerosis • cell adhesion molecules • endothelium • lipids

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Atherosclerosis is a chronic inflammatory disease of the arterial vessel wall that progresses from fatty streak to fibrofatty matrix and fibrous plaque. Monocyte recruitment to the vessel wall and transformation to lipid-enriched foam cells initiate and sustain atherosclerosis.1 12/15-Lipoxygenase (12/15-LO) is a nonheme iron-containing dioxygenase that forms 12-hydroperoxy-icosatetraenoic acid (12-HETE) and 15-HETE and oxidizes esterified fatty acids in lipoproteins (cholesteryl esters) and phospholipids.2,3 On the basis of its product (estrogen), it is classified as 15-lipoxygenase (15-LO) in humans and rabbits4,5 and as “leukocyte-type” 12-lipoxygenase (12-LO) in pig, rat, and mouse.6 12/15-LO may have different effects on atherosclerosis or other vascular diseases.7 It has been speculated that proatherogenic and antiatherogenic, which indicates a possibility that different cellular expression of 12/15-LO. In the present study, we used bone marrow transfer to determine the role of 12/15-LO in macrophages and vascular cells (including endothelial cells and smooth muscle cells) in the development of atherosclerosis. We

Pharmacological inhibition of 15-LO in hypercholesterolemic rabbits resulted in attenuation of atherosclerosis.8,9 In the apoE−/−, LDLR−/−, and apobec-1−/−/LDL-R−/− mouse models of atherosclerosis, disruption of the 12/15-LO gene significantly retarded the initiation and progression of atherosclerosis.10–12 A variety of vascular cells are able to express 12/15-LO, including endothelial cells,13,14 smooth muscle cells,13,14 and monocytes/macrophages.3,15 Overexpression of 12/15-LO in mouse endothelial cells16 and rabbit monocytes/macrophages17 resulted in completely opposite effects: The former was proatherogenic and the latter antiatherogenic, which indicates a possibility that different cellular expression of 12/15-LO may have different effects on atherosclerosis that species differences may exist.

In the present study, we used bone marrow transfer to determine the role of 12/15-LO in macrophages and vascular cells (including endothelial cells and smooth muscle cells) in the development of atherosclerosis. We

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also examined the effects of 12/15-LO on endothelial activation and monocyte adhesion using in vitro endothelium-monocyte interaction systems. Finally, we compared in vitro foam cell formation in bone marrow–derived and peritoneal macrophages isolated from 12/15-LO/−/− mice.

Methods

Mice: Bone Marrow Transplantation

The generation and genotype analysis of 12/15-LO−/−/apoE−/− mice have been described previously. Mouse bone marrow transplantation (BMT) was performed as described previously. Four weeks after recovery from BMT, mice were fed a Western diet for 12 weeks.

Determination of 12/15-LO mRNA Expression With Real-Time Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated with an RNeasy Mini Kit (Qiagen Inc). The primers used to analyze mRNA for mouse 12/15-LO were CTCTCAAGGCCTGTTCAGGA (sense) and GTCCATTGTCCCCAGACCT (antisense). Reverse transcription–polymerase chain reaction (RT-PCR) was performed on the iCycler (Bio-Rad Laboratories) with SYBR Green I (Molecular Probes).

Preparation of Mouse Aortas and Quantification of Atherosclerosis

The aortas of mice were collected and stained with oil red O. Images were scanned into a Macintosh computer, and the percent surface areas occupied by lesions were determined with Image-ProPlus (Media Cybernetics).

Measurement of Plasma Lipids, Isoprostanes, and Autoantibody Titers Against Oxidized LDL Epitopes

Plasma triglyceride and total cholesterol levels were determined via an automated enzymatic technique (Boehringer Mannheim GmbH). Plasma 8,12-iso-IPFα-VI levels were measured by gas chromatography/mass spectrometry. The titers of IgG and IgM autoantibodies against malondialdehyde LDL (MDA-LDL) and oxidized LDL (OxLDL) were analyzed as described previously.

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<tr>
<th>Donors</th>
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<td>apoE−/− mice</td>
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<td>12/15-LO in all expressing cells</td>
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Isolation and Culture of Murine Aortic Endothelial Cells and Macrophages

Endothelial cells from mouse thoracic aortas were isolated and cultured as described previously.21 Endothelial cells from passage 3 to 5 were used in this study. Macrophages used in this study were either harvested by peritoneal lavage or differentiated from bone marrow cell by cytokines that included interleukin-4 (IL-4), granulocyte-macrophage colony–stimulating factor (GM-CSF), and macrophage colony–stimulating factor (M-CSF).

Monocyte Adhesion Assay

Confluent murine endothelial monolayers were washed and cultured in serum-free DMEM supplemented with Nutridoma-HU (Roche Diagnostics GmbH). LDL (Biomedical Technologies, Inc) was added at a final concentration of 200 μg/mL. After 20 hours, cell media were removed, and the endothelial monolayer was washed 3 times for the monocyte adhesion assay. The confluent murine endothelial monolayer was cocultured with macrophages for 24 hours in serum-free DMEM supplemented with Nutridoma-HU in the presence of LDL at a concentration of 200 μg/mL. Then, washed endothelial monolayers were incubated with 10⁶ carboxyfluorescein diacetate succinimidyl ester-labeled WEHI78/24 cells (a murine monocytic cell line) suspended in 1 mL of binding buffer for 20 minutes. After a vigorous wash, adherent cells were determined by fluorescence intensity.

In Vitro Foam Cell Assay

Thioglycollate-elicited or bone marrow–derived macrophages were used. For some experiments, macrophages were stimulated with IL-4, LDL, OxLDL, or acetylated LDL (Biomedical Technologies, Inc) was added at different concentrations for different periods. Then, macrophages were washed with PBS, fixed with 4% paraformaldehyde, and stained with oil red O. Images were scanned into a Macintosh computer, and the percentage of oil red O–stained droplets or surface area occupied by oil red O–stained droplets in each cell was determined with Image-Pro Plus.

Statistical Analysis

Statistical analysis was performed with Instat software (GraphPad Software). Data are represented as mean±SE. Data were compared with either 1-way ANOVA followed by Bonferroni correction post hoc test or Student t test to evaluate 2-tailed levels of significance. The null hypothesis was rejected at P<0.05.

Results

Expression of 12/15-LO mRNA In Vivo

To investigate potential tissue-specific roles of 12/15-LO in atherosclerosis, we first surveyed 12/15-LO mRNA expression in atherosclerotic mice using real-time RT-PCR. Of all tissues surveyed, the highest mRNA expression level was found in peritoneal macrophages. 12/15-LO mRNA in elicited peritoneal macrophages was ∼1000 times higher than that in other tissues, including lung, heart, atherosclerotic vessel, liver, gut, spleen, lymph node, kidney, and adipose tissue. 12/15-LO was undetectable in brain and muscle (Figure 1). 12/15-LO was decreased by 90% in most tissues 12 weeks after 12/15-LO−/− bone marrow replacement (Figure 1), which suggests that 12/15-LO exists mainly in macrophages in these tissues. Alternatively, 12/15-LO expression of native cells in these tissues may be regulated by 12/15-LO–expressing macrophages, although no autocrine and paracrine loops of 12/15-LO expression have been reported. The amount of 12/15-LO mRNA in kidney and adipose tissues did not change significantly as a result of transplantation with 12/15-LO−/− bone marrow, which suggests 12/15-LO expression in tissue-resident cells. Under the conditions employed, 12/15-LO mRNA was not detectable in atherosclerotic arteries of apoE−/− mice that received 12/15-LO–deficient bone marrow, which indicates that 12/15-LO in atherosclerotic lesions exists mainly in macrophages/foam cells (Figure 1).

Role of Bone Marrow–Derived 12/15-LO in Atherosclerosis

Bone marrow–derived macrophages contribute to foam cell formation in atherosclerotic lesions.22 To determine
the influence of macrophage 12/15-LO in the formation of atherosclerotic lesions, we generated 4 groups of chimeric mice (Table) by transplanting bone marrow from 12/15-LO/H11002/ apoE/H11002 mice into apoE/H11002 mice and vice versa, as well as 2 control groups. BMT did not affect blood monocyte counts or tissue macrophages as reflected by the number of macrophages in the peritoneal cavity of thioglycollate-challenged mice (data not shown). Aortic lesion sizes in the 4 groups are shown in Figure 2b. ApoE/H11002/mice reconstituted with bone marrow of apoE/H11002/mice had lesions that covered 4.5% to 8.7% of the aortic surface. The lesion size in apoE/H11002/mice receiving the bone marrow of 12/15-LO/H11002/ apoE/H11002 mice, similar to that in 12/15-LO/H11002/ apoE/H11002 mice receiving the bone marrow of 12/15-LO/H11002/ apoE/H11002, was reduced by 50% (2.0% to 4.2%) compared with that in their controls (Figure 2b). This suggests that 12/15-LO from bone marrow–derived cells is critical for lesion development in this model. Consistent with this interpretation, reconstitution of 12/15-LO/H11002/ apoE/H11002 mice with bone marrow of apoE/H11002 mice fully restored their lesion sizes to the levels of apoE/H11002 mice.

Plasma Lipids, Lipid Peroxidation, and Immune Response in Reconstituted apoE/−/− Mice
Mean cholesterol and triglyceride levels in bone marrow–transferred mice in different groups were not different (data not shown). Because the apoE/−/− mice reconstituted with bone marrow from 12/15-LO/−/−/ apoE/−/− mice had small atherosclerotic lesions, indistinguishable from the lesion size found in mice completely lacking 12/15-LO, we tested whether plasma 8,12-iso-iPF$_{2alpha}$-VI levels in these mice would also be suppressed. Interestingly, isoprostane levels in these mice were the same as in apoE/−/− or 12/15-LO/−/−/apoE/−/− mice reconstituted with the bone marrow of apoE/−/− mice (Figure 3a).

In atherosclerosis, an immune response to oxidized lipids is prominent and results in the formation of autoantibodies of the IgM and IgG isotypes to modified LDL. In all groups of BMT mice, autoantibody levels, especially levels of IgG autoantibodies, were much lower than those of age-matched controls, presumably because the immune system had not completely recovered from the lethal irradiation and reconstitution at the time serum was harvested. Interestingly, autoantibody (IgM Ab against
copper-modified LDL) levels were higher in mice reconstituted with the bone marrow of apoE<sup>−/−</sup> mice than in those receiving the bone marrow of 12/15-LO<sup>−/−</sup>/apoE<sup>−/−</sup> mice, irrespective of the genotype of the recipient (Figure 3b). Consistent with protection of mice receiving 12/15-LO<sup>−/−</sup>/apoE<sup>−/−</sup> bone marrow, we found a highly significant positive correlation of autoantibody (IgM Ab against copper-modified LDL) levels with lesion size (Figure 3c). Autoantibody levels did not correlate with levels of plasma isoprostanes (Figure 3d).

**Endothelial Activation by Autocrine 12/15-LO Products**

Aortic endothelium of apoE<sup>−/−</sup> mice fed a Western diet is activated and expresses various adhesion molecules. Because endothelial cells are known to express 12/15-LO, we tested whether endothelial activation is caused by endothelial cell 12/15-LO by an autocrine mechanism. Figure 4 shows that there was no difference in the expression of either vascular cell adhesion molecule-1 (VCAM-1) or intercellular adhesion molecule-1 (ICAM-1) between cultured wild-type and 12/15-LO<sup>−/−</sup> aortic endothelial cells, either incubated with LDL, stimulated with tumor necrosis factor-α, or under resting conditions.

Similarly, adhesion of WEHI78/24 cells, a murine monocytic cell line, also was not dependent on endothelial 12/15-LO (Figure 4a).

**Endothelial Activation by Macrophage 12/15-LO Activity**

To test whether macrophage 12/15-LO has an influence in endothelial activation and endothelium-monocyte interactions, we developed a coculture system (Figure 5a) in which peritoneal macrophages from 12/15-LO<sup>−/−</sup> or wild-type mice were incubated in the upper well of a transwell system with and without native (nonmodified) LDL, and endothelial cells in the lower well were used as indicators of endothelial activation, measured by adhesion of WEHI78/24 cells. In the absence of LDL, neither wild-type nor 12/15-LO<sup>−/−</sup> macrophages had a large and significant activating effect on endothelial cells, as demonstrated by a 3-fold elevation of WEHI78/24 cell adhesion (Figures 5b and 5c) and significant upregulation of VCAM-1 on endothelial cells (Figures 5d and 5e).
Foam Cell Formation by 12/15-LO–Deficient Macrophages

Foam cell formation is a hallmark of atherosclerosis. Therefore, we tested whether 12/15-LO might influence foam cell formation in peritoneal macrophages loaded with acetylated LDL. After 24 and 48 hours in culture, oil red O uptake as a marker of lipid accumulation was indistinguishable in macrophages from wild-type and 12/15-LO/H11002/H11002 mice (Figure 6a). Similar results were obtained with OxLDL (data not shown).

When 12/15-LO–deficient peritoneal macrophages were cultured in the presence of native LDL, their lipid uptake was much reduced compared with wild-type controls. The intensity of oil red O staining was decreased in peritoneal macrophages from 12/15-LO/H11002/H11002 mice (Figures 6b and 6c). This difference was further enhanced in macrophages stimulated with IL-4 (Figures 6b and 6c). This may be due to an increase of 12/15-LO protein and activity induced by IL-4 in vitro. Similar results were obtained on macrophages differentiated from bone marrow cells in the presence of GM-CSF, IL-4, and M-CSF (data not shown).

Discussion

Our study demonstrates that 12/15-LO in macrophages is critical for the formation of atherosclerotic lesions. Mice reconstituted with bone marrow from apoE/H11002/H11002 mice show >50% reduced lesion sizes compared with mice receiving apoE/H11002 bone marrow. Double-knockout mice regained the severity of atherosclerotic lesion typical of apoE/H11002 mice after receiving bone marrow from apoE/H11002 mice. Thus, macrophage 12/15-LO mediates endothelial activation and foam cell formation, 2 key steps in the process of atherosclerosis.

The BMT study suggests that macrophage 12/15-LO is proatherogenic in mice. This is inconsistent with previous studies by Shen et al.,17 who found that 15-LO in monocytes/macrophages is antiatherogenic in a rabbit atherosclerotic model. The conflicting conclusions from these studies may be due to differences in species (rabbit versus mouse), lipoxygenase gene products (ratio of 12HETE:15HETE), and genetic manipulation (knockout versus transgenic overexpression). The expression level of the enzyme in transgenic monocyte-derived rabbit macrophages is >20-fold higher than in macrophages of normal rabbits and comparable to that of highly activated human monocytes by cytokines.27 12/15-LO and its products at high concentrations may play a role different from that at their physiological concentration. Recent studies have shown that several 12/15-LO products are ligands of peroxisome proliferator–activated receptor-γ (PPARγ).28 PPARγ ligands have potent antiinflammatory effects at a high concentration.29 Overexpression of 15-LO in monocytes/macrophages may result in substantial accumulation of 12/15-LO products and consequently trigger the PPARγ pathway in these cells.

Circulating precursor cells can be recruited to the vessel wall and differentiate to various vascular cells under different pathological conditions, especially in the setting of vascular injury.30 In spontaneous atherosclerotic vessels, only a very limited number of endothelial cells and smooth muscle cells are replaced by differentiated donor-derived stem cells within 8 to 12 weeks after BMT.30 In the same time frame, 12/15-LO/H11002/ apoE/H11002 mice developing bone marrow from apoE/H11002 mice developed the same extent of atherosclerotic lesions as control mice. There-
fore, the contribution of circulating precursor cells to the observed effects, if there is any, is likely to be small.

Human monocytes and macrophages express a 15-LO that can also produce 12-HETE and probably represents the human orthologue of mouse 12/15-LO. The presence of specific 15-lipoxygenase products, 15-LO protein and 15-LO mRNA in human atherosclerotic arteries has been clearly demonstrated in samples obtained from patients aged 15 to 37 years. In a recent report, Spanbroek et al did not detect 15-LO in very advanced atherosclerotic arterial samples. The present study was performed at the time when mice start to develop atherosclerosis, showing participation of macrophage 12/15-LO in the early phase of atherogenesis in mice, which is consistent with the findings in humans.

In the absence of LDL, macrophage 12/15-LO causes little increase in endothelial activation. However, in the presence of LDL, macrophage 12/15-LO significantly increases expression of endothelial adhesion molecules and endothelial-monocyte interactions. A suppression of lipid uptake and foam cell formation in macrophages lacking 12/15-LO was observed after incubation with native LDL but not modified LDL. These results suggest that the proatherogenic role of macrophage 12/15-LO may be related to its oxidative action on LDL. However, in this BMT study, levels of isoprostane do not correlate with the sizes of atherosclerotic lesions. Overall levels of isoprostane may not reflect the local accumulation of oxidative products generated by 12/15-LO in the vessel wall, which may be important for the formation of atherosclerotic lesions. Alternatively, other 12/15-LO–dependent mechanisms may be involved. A recent study has demonstrated a decrease in production of IL-12 in 12/15-LO–deficient macrophages, which suggests a reduced immune response. This correlates with lower titers of autoantibodies to OxLDL in BMT mice in the present study. Therefore, in addition to its oxidative action, macrophage-derived 12/15-LO may also be involved in regulating the immune response during initiation and progression of atherosclerosis.

Acknowledgments

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/content/110/19/3156.4.full.pdf

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Corrections

In the article “Omega-3 Fatty Acids in Cardiac Biopsies From Heart Transplantation Patients: Correlation With Erythrocytes and Response to Supplementation,” by Harris et al, which appeared in the September 21, 2004, issue of the journal (Circulation. 2004;110:1645–1649), the authors have identified 2 errors. On page 1646, under the heading “Plasma Lipids and Lipoproteins,” the abbreviation in parentheses on the third line should be HDL-C, not LDL-C. Also, in the final line in column 1 of page 1648, the word “not” was omitted. The sentence should read, “We found that RBC EPA+DHA was not altered in postprandial blood from 10 healthy volunteers…” The authors regret these errors.

Regarding the Correspondence page “Dietary Intervention Combined With Exercise Improves Vascular Dysfunction but Also Obstructive Sleep Apnea in Obese Children,” which appeared in the September 21, 2004 issue of the journal (Circulation. 2004;110:e314), it has been brought to our attention that this title is misleading. The 2 letters on this page pertained to an article published in the April 27, 2004, issue of the journal by Woo et al (Circulation. 2004;109:1981–1986) titled “Effects of Diet and Exercise on Obesity-Related Vascular Dysfunction in Children.” The second letter (available at http://circ.ahajournals.org/cgi/content/full/110/12/e314), written by Tsung O. Cheng, should have been titled “Childhood Obesity Among the Chinese.” We regret this error.

The article “Antibodies From Preeclamptic Patients Stimulate Increased Intracellular Ca$^{2+}$ Mobilization Through Angiotensin Receptor Activation” by Thway et al, which appeared in the September 21, 2004, issue of the journal (Circulation. 2004;110:1612–1619), was inadvertently published without the authors’ corrections. The corrected version is available online at http://circ.ahajournals.org/cgi/content/full/110/12/1612. We regret these errors.

In the article “Critical Role of Macrophage 12/15-Lipoxygenase for Atherosclerosis in Apolipoprotein E-Deficient Mice” by Huo et al that appeared in the October 5, 2004, issue of the journal (Circulation. 2004;110:2024–2031), an error appeared in the legend of Figure 4. The following sentences should have been deleted: d, Serum-free cell medium conditioned by 12/15-LO$^{+/+}$ macrophages slightly increased endothelial VCAM-1 expression. e, In presence of LDL, 12/15-LO$^{+/+}$ macrophages cocultured with endothelial cells significantly increased expression of VCAM-1 on endothelial cells. n=4.