We have shown that the 12/15-lipoxygenase (12/15-LO) product 12S-hydroxyeicosatetraenoic acid increases monocyte adhesion to human endothelial cells (EC) in vitro. Recent studies have implicated 12/15-LO in mediating atherosclerosis in mice. We generated transgenic mice on a C57BL/6J (B6) background that modestly overexpressed the murine 12/15-LO gene (designated LOTG). LOTG mice had 2.5-fold elevations in levels of 12S-hydroxyeicosatetraenoic acid and a 2-fold increase in expression of 12/15-LO protein in vivo. These mice developed spontaneous aortic fatty streak lesions on a chow diet. Thus, we examined effects of 12/15-LO expression on early events leading to atherosclerosis in these mice. We found that, under basal unstimulated conditions, LOTG EC bound more monocytes than B6 control EC (18 ± 2 versus 7 ± 1 monocytes/field, respectively; p < 0.0001). Inhibition of 12/15-LO activity in LOTG EC using a 12/15-LO ribozyme completely blocked monocyte adhesion in LOTG mice. Thus, 12/15-LO activity is required for monocyte/EC adhesion in the vessel wall. Expression of ICAM-1 in aortic endothelia of LOTG mice was increased severalfold. VCAM-1 expression was not changed. In a series of blocking studies, antibodies to α4 and β2 integrins in WEHI monocytes blocked monocyte adhesion to both LOTG and B6 control EC. Inhibition of ICAM-1, VCAM-1, and connecting segment-1 fibronectin in EC significantly reduced adhesion of WEHI monocytes to LOTG EC. In summary, these data indicate that EC from LOTG mice are “pre-activated” to bind monocytes. Monocyte adhesion in LOTG mice is mediated through β2 integrin and ICAM-1 interactions as well as through VLA-4 and connecting segment-1 fibronectin/VCAM-1 interactions. Thus, 12/15-LO mediates monocyte/EC interactions in the vessel wall in atherogenesis at least in part through molecular regulation of expression of endothelial adhesion molecules.

Harats et al. (11) found that significant overexpression of the human 15-LO gene in vascular endothelial cells (EC) increases aortic atherosclerosis. The mechanisms by which 12/15-LO products cause atherosclerosis remain unknown, yet may relate to altered inflammatory pathway signaling by 12/15-LO products in both the endothelium and monocytes/macrophages in the vessel wall. Indeed, Zhao et al. (12) have recently found that macrophages from 12/15-LO knockout mice have reduced synthesis of interleukin-12. Other mechanisms contributing to the atherogenicity of 12/15-LO include the enzyme's ability to oxidize lipids and lipoproteins in the vasculature and the ability of its eicosanoid products to mediate monocyte adhesion to the endothelium. Several groups have shown that the human 15-LO enzyme oxidizes LDLs in vitro and that 12/15-LO inhibits LDL oxidation, suggesting that the enzyme's ability to oxidize lipids and lipoproteins may contribute to atherogenesis.

1 The abbreviations used are: 12/15-LO, 12/15-lipoxygenase; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; LDL, low density lipoprotein; EC, endothelial cell(s); VLA-4, very late antigen-4; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; CS-1, connecting segment-1; GC, cin- namily-3,4-dihydroxy-α-cyanoacetaminic acid; LOTG, 12/15-LO transgenic; HPLC, high performance liquid chromatography; FITC, fluorescein isothiocyanate; LORO, 12/15-LO knockout; MAEC, mouse aortic endothelial cells; TNF-α, tumor necrosis factor-α; MOPS, 4-morpholinosopropane-sulfonic acid; GFP, green fluorescent protein; ANOVA, analysis of variance; FN, fibronectin.
The 12/15-lipoxygenase (LO) pathway involves the production of eicosanoids, which are involved in various biological processes, including inflammation and the formation of atherosclerotic plaques. The 12/15-LO enzyme was excised from the FIXII vector using the NotI restriction sites. This generated a linear genomic clone that included 7 kb downstream of the 4.5-kb murine 12/15-LO gene. Exons 1–14 were included in the genomic clone using NotI allowed inclusion of the T7 and T3 sequences that were used in the screening strategy to identify positive founder mice. Identification of two founder lines (16F and 41F) was achieved using PCR. PCR was performed on genomic DNA isolated from rodent tail clips as described under “Experimental Procedures.” Shown are a 333-bp PCR product using the T3/T3271 primers and a 407-bp product using the T7/T7409 primers. The NotI-excised genomic clone was analyzed as a positive control by PCR for identification purposes. Southern analysis of genomic DNA isolated from both transgenic founder lines was performed on a 1% agarose gel. The genomic DNA was probed with a 32P-labeled full-length mouse 12/15-LO cDNA probe. Ladder bands were obtained from Molecular Probes, Inc.

**Generation of Mice—** 12/15-Lipoxygenase transgenic mice (designated LOTG) were generated on a C57BL/6J (B6) background using a 15.5-kb mouse genomic clone as shown in Fig. 1A. This genomic clone contains the full-length murine 4.5-kb leukocyte-type 12-LO gene (4). The genomic clone also comprises ~7 kb upstream and 2 kb downstream of the murine 12/15-LO gene to include additional proximal promoter and enhancer elements. The genomic clone was excised from the FIXII (Stratagene) using NotI and injected into C57BL/6J mouse blastocysts. Two founder lines were generated: 16F and 41F. Mice from both founder lines were viable and fertile. We have not observed phenotypic differences between male and female LOTG mice. The genotyping for the founder mice using the T3/T3271 and T7/T7409 primers was performed.

**EXPERIMENTAL PROCEDURES**

**Reagents—** Rat anti-mouse antibodies to VLA-4 (PS2; American Type Culture Collection) and VCAM-1 (MK2/7; American Type Culture Collection) were provided by Dr. Klaus Ley (University of Virginia). Phycoerythrin-labeled anti-mouse NK1.1 antibody (PK136) was purchased from BD Biosciences. NycoPrep one-step 1.077/265 animal separation solution was purchased from Accurate Chemical Co. Connecting segment-1 (CS-1) peptide (EILDVFPST) was purchased from American Peptide Co. Affinity-purified antibody to 12/15-LO was provided by Dr. Jiali Gu (University of Virginia). WEHI 78/24 monocyte cells were a gift of Dr. Judith A. Berliner (UCLA). Cinnamyl-3,4-dihydroxy-cyanocinnamate (CDC), a lipoxygenase inhibitor, was obtained from BIOMOL Research Labs Inc. Calcein O,O′-diacetate tetrakis(acetoxymethyl) ester was obtained from Molecular Probes, Inc.

To understand the mechanisms by which 12/15-LO-generated eicosanoids modulate events leading to atherogenesis *in vivo*, we produced transgenic mice that modestly overexpressed the murine 12/15-LO gene. In *vivo*, these mice produced 2-fold elevations in levels of 12S-HETE and 13S-HODE, the major eicosanoid products of the enzyme. The transgenic mice developed spontaneous aortic fatty streak lesions on a rodent chow diet. Thus, we examined the role of 12/15-LO activity in mediating monocyte/endothelial interactions, key early events in atherogenesis *in vivo* (20).

**RESULTS**

**A.** Schematic of the genomic construct used to generate LOTG mice. The genomic clone was excised from the FIXII vector using the NotI restriction sites. This generated a linear genomic clone that included ~7 kb upstream and 2 kb downstream of the 4.5-kb murine 12/15-LO gene. Exons 1–14 are illustrated. Excision of the genomic clone using NotI allowed inclusion of the T7 and T3 sequences that were used in the screening strategy to identify positive founder mice. Identification of two founder lines (16F and 41F) was achieved using a PCR strategy. PCR was performed on genomic DNA isolated from rodent tail clips as described under “Experimental Procedures.” Shown are a 333-bp PCR product using the T3/T3271 primers and a 407-bp product using the T7/T7409 primers. The NotI-excised genomic clone was analyzed as a positive control by PCR for identification purposes. Southern analysis of genomic DNA isolated from both transgenic founder lines was performed on a 1% agarose gel. The genomic DNA was probed with a 32P-labeled full-length mouse 12/15-LO cDNA probe. Ladder bands were obtained from Molecular Probes, Inc.

**FIG. 1. Generation of murine 12/15-LO transgenic mice.** A, schematic of the genomic construct used to generate LOTG mice. The genomic clone was excised from the FIXII vector using the NotI restriction sites. This generated a linear genomic clone that included ~7 kb upstream and 2 kb downstream of the 4.5-kb murine 12/15-LO gene. Exons 1–14 are illustrated. Excision of the genomic clone using NotI allowed inclusion of the T7 and T3 sequences that were used in the screening strategy to identify positive founder mice. B, identification of two founder lines (16F and 41F) using a PCR strategy. PCR was performed on genomic DNA isolated from rodent tail clips as described under “Experimental Procedures.” Shown are a 333-bp PCR product using the T3/T3271 primers and a 407-bp product using the T7/T7409 primers. The NotI-excised genomic clone was analyzed as a positive control by PCR for identification purposes. C, Southern analysis of genomic DNA isolated from both transgenic founder lines. Genomic DNAs from B6 control and 16F and 41F transgenic mice were separated on a 1% agarose gel. The genomic DNA was probed with a 32P-labeled full-length mouse 12/15-LO cDNA probe. Ladder bands were obtained from Molecular Probes, Inc.
T7409 primers were shown in Fig. 1B. From the founder mice, these PCR products were sequenced and found to correspond to the 12/15-LO genomic clone sequence. Southern analysis of genomic DNA from C57BL/6J control mice and 16F and 41F transgenic mice showed increased 12/15-LO copy number in both transgenic lines compared with the control line (Fig. 1C). The full-length mouse 12/15-LO cDNA was used as a probe for Southern analysis. In some studies, we used 12/15-LO knockout mice on a C3H/HeJ background (21). These mice were a kind gift of Dr. Colin D. Funk (University of Pennsylvania).

**Eicosanoid Measurement in Urine**—Lipids were extracted from urine as described previously using Varian C8 Bond-Elut columns in series (10). 8S-HETE was added as an internal standard. The fluorescence derivatives of the free fatty acids were formed using 8 mg of 2-(2,3-naphthalenyl)-5-methyl-3H-tetrazolium (NBT) and 0.1% acetic acid, and solvent A (0.1% acetic acid) at a flow rate of 1.2 ml/min following related protocols of Roman and co-workers (22). Peaks were detected fluorometrically at an excitation wavelength of 259 nm and an emission wavelength of 394 nm. The area ratio of sample HETE area to internal standard (8S-HETE) area was plotted against nanograms of HETE injected, and unknown HETE values were calculated from their area ratios. HETEs (5,12,15-HETE) and HODEs (9,13-HODE) were baseline-separated as described previously (22). Peaks were detected using a BD Biosciences FACSVantage SE™ cell sorter by for - mulation of a prostate inhibitor mixture (Sigma). 75 µg of total EC protein was analyzed by 4–12% SDS-PAGE in MOPS running buffer (Invitrogen) and transferred to nitrocellulose. Pierce BLOTTO buffer was used as a blocking agent. Membranes were probed with a 1:1000 dilution of rabbit polyclonal antibody to murine 12/15-LO. 12/15-LO protein was detected using a 1:2000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG and chemiluminescence. Bands were normalized to tubulin (15000 dilution of antibody) and quantitated by densitometry.

**Mouse Monocyte Isolation**—Whole blood was obtained via retro-or bital plexus puncture. Whole blood was collected into heparinized syringes and 15 heterozygous CX3CR1-GFP mice. These mice were generated by replacing the coding region of CX3CR1 (the fractalkine receptor) with GFP. Thus, these mice express GFP only in monocytes and in a subset of natural killer cells. GFP expression in the monocytes allowed us to purify monocytes using sterile fluorescence cell sorting. To isolate the monocytes, whole blood was collected into hepa rin and diluted with an equal volume of 0.9% saline. 6 ml of diluted blood was carefully layered over 3 ml of Nycodenz one-step 1.077/265 g/ml. The density gradient was centrifuged at 400 x g for 30 min at 4°C. Unbound monocytes were harvested in 1 ml of Hanks’ balanced salt solution, centrifuged at 400 x g for 5 min, and resuspended in 1 ml of Hanks’ balanced salt solution. For cell sorting, monocytes were incubated for 30 min at 4°C with allopurinol and labeled anti-NK1.1 antibody to label natural killer cells. After incubation, cells were rinsed with Hanks’ balanced salt solution, resuspended in 1 ml of Hanks’ balanced salt solution, and sorted using a BD Biosciences FACSVantage SE™ cell sorter by for - mulation of a prostate inhibitor mixture (Sigma). 75 µg of total EC protein was analyzed by 4–12% SDS-PAGE in MOPS running buffer (Invitrogen) and transferred to nitrocellulose. Pierce BLOTTO buffer was used as a blocking agent. Membranes were probed with a 1:1000 dilution of rabbit polyclonal antibody to murine 12/15-LO. 12/15-LO protein was detected using a 1:2000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG and chemiluminescence. Bands were normalized to tubulin (15000 dilution of antibody) and quantitated by densitometry.

**Flow Cytometry Analysis of Endothelial Adhesion Molecule Expression**—Mouse EC at passage 4 were collected in phosphate-buffered saline by gentle scraping using a cell scraper. Cells (150,000/sample) were analyzed for each antibody. Cells were incubated for 30 min at 4°C with a 1:100 dilution of fluorescent isothiocyanate (FITC)-labeled primary antibodies for mouse adhesion molecules (FITC-labeled anti-mouse ICAM-1, BD Biosciences catalog no. 553225; and FITC-labeled anti-mouse VCAM-1, BD Biosciences catalog no. 553332) with 0.1% acetic acid, and solvent A (0.1% acetic acid) at a flow rate of 1.2 ml/min following related protocols of Roman and co-workers (22). Representative HPLC chromatograms from B6 control and LOTG mice (panel B).

**Aortic Atherosclerosis Measurements**—Male C57BL/6J mice and male mice from the 1F line (8 weeks old) were fed a rodent chow diet for 20 weeks. After 20 weeks, mice were euthanized. The organs were collected in the FL1 channel through a 530/30-nm band-pass filter. Oxidized LDL was prepared as described previously using Varian C18 Bond-Elut columns in series (6). The full-length mouse 12/15-LO cDNA was used to form an internal standard. The fluorescence derivatives of the free fatty acids were formed using 8 mg of 2-(2,3-naphthalenyl)-5-methyl-3H-tetrazolium (NBT) and 0.1% acetic acid, and solvent A (0.1% acetic acid) at a flow rate of 1.2 ml/min following related protocols of Roman and co-workers (22). Peaks were detected fluorometrically at an excitation wavelength of 259 nm and an emission wavelength of 394 nm. The area ratio of sample HETE area to internal standard (8S-HETE) area was plotted against nanograms of HETE injected, and unknown HETE values were calculated from their area ratios. HETEs (5,12,15-HETE) and HODEs (9,13-HODE) were baseline-separated as described previously (22). Peaks were detected using a BD Biosciences FACSVantage SE™ cell sorter by formulation of a prostate inhibitor mixture (Sigma). 75 µg of total EC protein was analyzed by 4–12% SDS-PAGE in MOPS running buffer (Invitrogen) and transferred to nitrocellulose. Pierce BLOTTO buffer was used as a blocking agent. Membranes were probed with a 1:1000 dilution of rabbit polyclonal antibody to murine 12/15-LO. 12/15-LO protein was detected using a 1:2000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG and chemiluminescence. Bands were normalized to tubulin (15000 dilution of antibody) and quantitated by densitometry.

**Quantitative Real-time PCR for Murine ICAM-1 and Murine 12/15- LO**—Organ tissues were collected from control and LOTG mice from the 41F line and quick-frozen in liquid nitrogen for 12/15-LO mRNA measurements. MAEC were freshly isolated from aortas and cultured as described above in 100-mm cell dishes for 12/15-LO mRNA and ICAM-1 mRNA measurements. Total cellular RNA was obtained from either MAEC or frozen tissue using TRIzol (Invitrogen) according to the manufacturer’s instructions. Reverse transcription of 2 µg of total RNA was performed in a total volume of 25 µl using ThermoScript RT (Invitrogen). This reaction was then processed to remove the DNAase treatment temperature for 10 min. The reaction was stopped by addition of EDTA. For quantitative PCR analysis, the resulting cDNA was diluted 1:5, and 2 µl was used for each PCR. Reagents from the QIAGEN real-time PCR kit containing SYBR Green were used for quantitative PCRs. The PCR conditions for ICAM-1 were as follows: 95°C for 10 min; 95°C for 4 min; 55°C for 15 s; 72°C for 5 s; followed by a final extension at 81°C for 15 s. For 12/15-LO and cyclophilin, the PCR conditions were as follows: 95°C for 10 min; 95°C for 4 min; followed by 40 cycles at 95°C for 15 s, 62°C for 30 s, and 72°C for 30 s; followed by a final extension at 81°C for 15 s. The primers for murine 12/15-LO were 5'-cttccaaggctggtc-3' (forward) and 5'-agcatcaagttctc-3' (reverse). The primers for murine cyclophilin were 5'-tgagaacagacaacagac-3' (forward) and 5'-tgccggtgtagcagatg-3' (reverse). Data were analyzed and are presented based upon the relative...
Fig. 2. HPLC analysis of eicosanoid production in 12/15-LO transgenic mice. Urine was collected from mice using metabolic cages, and HPLC analysis of eicosanoid production was performed as described under "Experimental Procedures." A, an HPLC chromatogram illustrating elution profiles of a standard mixture of HETEs ($He$) and HODEs ($Ho$). B, representative elution profiles of eicosanoids present in the urine of a B6 control mouse and a LOTG mouse.
expression method (31). This formula for calculation is follows: relative expression = $2^{\Delta Ct}$, where $\Delta Ct$ is the difference in threshold cycle between the gene of interest (either 12/15-LO or ICAM-1) and the housekeeping gene (cyclophilin), S is LOTG mice, and C is B6 control mice.

Statistical Analyses—Comparisons between groups were performed by analysis of variance (ANOVA). Data are represented as the means ± S.E. of eight mice (unless otherwise noted in the figure legends). All comparisons were made using Fisher’s Least Significant Difference procedure so that multiple comparisons were made at the 0.05 level only if the overall F-test from ANOVA was significant at $p < 0.05$.

RESULTS

**12/15-LO Transgenic Mice Have Increased Levels of 12S-HETE and 13S-HODE in Vivo**—LOTG mice had significant elevations in levels of urinary 12S-HETE and 13S-HODE, the primary eicosanoid products of the 12/15-LO enzyme (Fig. 4). Levels of 12S-HETE were increased from 200 ng/g of creatinine in B6 control mice to 500 ng/g of creatinine in LOTG mice (41F line) ($p < 0.0001$). Levels of urinary 13S-HODE were found to be 10 ng/g of creatinine in B6 control mice and 50 ng/g of creatinine in LOTG mice (41F line) ($p < 0.0001$). Levels of urinary 12S-HETE and 13S-HODE were also elevated in mice from the 16F line (289 ng/g of creatinine for 12S-HETE and 17 ng/g of creatinine for 13S-HODE; $p < 0.001$), although the increase in eicosanoid production in this transgenic line was much more modest. Overall, there was no apparent shunting to the 5-LO pathway in the LOTG mice, as 5S-HETE levels in LOTG mice remained similar to B6 control levels. We have previously reported that 5S-HETE does not induce monocyte adhesion to the endothelium (20). Tissue distribution of 12/15-LO mRNA in LOTG mice was normal, with expression found in aorta, brain, kidney, liver, and pancreas. Expression of 12/15-LO mRNA was increased 2-fold in aorta, liver, and pancreas and 3–4-fold in brain, heart, and kidney of LOTG mice compared with B6 mice (Fig. 5).

**Endothelial Cells from 12/15-LO Transgenic Mice Are “Preactivated” to Bind Monocytes**—We have shown that nanomolar concentrations of 12S-HETE activate human aortic EC to bind monocytes (20). Based upon these initial data in human aortic EC, we wondered if aortic EC from LOTG mice would show increased monocyte adhesion. We recently developed a technique in the laboratory for isolation of primary MAEC (24). EC were isolated from B6 and LOTG mice and used under basal unstimulated conditions. As shown in Fig. 6, there was a significant increase in monocyte adhesion to unstimulated LOTG EC from both founder lines (4 ± 1 monocytes/field for 16F mice and 7 ± 1 monocytes/field for 41F mice versus 2 ± 1 monocytes/field for B6 control mice; $p < 0.01$). In this experiment, we also compared adhesion of WEHI 78/24 cells (a mouse monocyte cell line) with that of primary mouse monocytes. Mouse peripheral blood monocytes are difficult to isolate, so we routinely use the WEHI mouse monocytes in adhesion assays. Both WEHI monocyte binding and mouse peripheral blood monocyte binding to EC were higher in LOTG mice than in B6 control mice ($p < 0.001$) (Fig. 6). These data indicate that 1) unstimulated basal EC isolated from LOTG mice from both founder lines displayed increased adhesion; 2) WEHI 78/24 cells bound B6 control and LOTG EC in a similar manner compared with primary mouse monocytes, although the background adhesion was slightly higher with the WEHI cells; and 3) the 41F line showed significantly higher adhesion than the 16F line, presumably due to the slightly higher activity of 12/15-LO in this line (Figs. 1, 2, 4, and 5). Thus, based upon adhesion data and the eicosanoid levels in the 16F and 41F lines, we chose to examine monocyte/endothelial interactions and atherosclerosis in the higher expressing 41F line only in all subsequent experiments.

**Aortic Endothelial Cells from 12/15-LO Transgenic Mice Have Increased 12/15-LO—Using primers specific for murine 12/15-LO in a quantitative PCR, we found a 3-fold induction of 12/15-LO mRNA in aortic EC from LOTG mice (line 41F) compared with B6 control mice (Fig. 7). In addition, we found a coordinated induction of 12/15-LO protein in aortic EC from LOTG mice (line 41F) compared with B6 control mice ($p < 0.009$) (Fig. 7). These data reflect a 2.5-fold increase in 12/
15-LO protein expression in aortic EC from LOTG mice of the 41F line.

12/15-LO Transgenic Mice Develop Aortic Atherosclerosis on a Chow Diet—Male mice from the 41F line were fed a chow diet for 15 weeks. Aortas were isolated from the heart to the iliac artery (3 mm from the bifurcation) and quantified for atherosclerosis by en face staining of the aorta according to the methods of Daugherty and Whitman (23). Laboratory strains of mice do not develop significant aortic fatty streak lesions when maintained on chow diets (33–35). LOTG mice developed spontaneous aortic atherosclerotic lesions on a chow diet (Fig. 8). The lesions resembled typical fatty streaks with subendothelial accumulations of lipid and were most prominent around the aortic arch and intercostal areas (data not shown).

Decreased Monocyte Adhesion to Endothelial Cells from 12/15-LO Knockout Mice—We also isolated EC from LOKO mice, which are on a C57BL/6J background and have a global deletion of the murine 12/15-LO gene (5, 21). We examined monocyte adhesion to LOKO EC and to EC that were stimulated with 250 g/ml mildly oxidized LDL for 4 h. As shown in Fig. 9, WEHI monocyte adhesion to EC from LOKO mice was significantly lower compared with B6 control or LOTG mice from line 41F (3 monocytes/field for LOKO mice versus 6 monocytes/field for B6 control mice versus 18 monocytes/field for LOTG mice; p < 0.0001). All EC groups responded to oxidized LDL, although LOTG EC bound more monocytes than B6 control or LOKO EC in all cases. TNF-α was used as a positive control to indicate maximal binding in the adhesion assay.

12/15-LO Products Directly Stimulate Monocyte/Endothelial Adhesion—To test the hypothesis that 12/15-LO activity directly mediates monocyte adhesion, we inhibited expression of 12/15-LO in LOTG EC using an adenovirus expressing a ribozyme to 12/15-LO as well as the pharmacological 12/15-LO inhibitor CDC and then measured adhesion. CDC blocks platelet 12-LO and 12/15-LO expression in EC (36). The DNA/RNA hammerhead ribozyme was generated to recognize the first 7 bp of the porcine and murine 12/15-LO mRNA sequences (30).
12/15-Lipoxygenase and Monocyte/Endothelial Interactions

We inserted the ribozyme into an adenoviral vector for transfection into primary EC and have previously used this adenoviral ribozyme construct to block 12/15-LO expression in porcine EC (30). Blocking the 12/15-LO pathway in LOTG mice using the 12/15-LO ribozyme or CDC significantly reduced monocyte adhesion (Fig. 10). To also test the hypothesis that 12/15-LO products can directly stimulate monocyte adhesion, B6 control and LOKO EC were treated for 4 h with 100 nM 12S-HETE and then used in an adhesion assay. This concentration of 12S-HETE is believed to be within the physiological range found in blood (see “Discussion”) (37–42). We performed dose-response studies with exogenous 12S-HETE on monocyte/EC adhesion and found, within a dose range of 1–500 nM 12S-HETE, that 100 nM 12S-HETE was the lowest concentration that provided a significant increase in monocyte adhesion to B6 control EC (data not shown). As shown in Fig. 11, addition of 100 nM 12S-HETE to B6 control and LOKO EC significantly increased monocyte adhesion. The enantiomer 12R-HETE, which is not a product of the 12/15-LO pathway, had no effect on monocyte adhesion. Taken together, these data illustrate that products of the 12/15-LO enzyme are primary mediators of monocyte/endothelial interactions in the vessel wall.

**DISCUSSION**

Recent studies have defined a role for 12/15-LO products in mediating inflammation and atherosclerosis (7–9, 11–13, 21, 27, 46, 47). However, the mechanisms through which these 12/15-LO eicosanoids act to mediate inflammation and atherosclerosis are still not well understood. Generation of 12/15-LO knockout mice by Funk and co-workers has contributed significantly to our understanding of 12/15-LO (5–7, 9, 12, 21, 32). Often, however, deletion of a key arachidonic acid pathway in mice can result in shunting of arachidonic acid into other eicosanoid pathways, as is the case with 12/15-LO knockout mice (32), thus making data interpretation somewhat difficult. To define the mechanisms by which 12/15-LO activity mediates...
atherosclerosis, we generated transgenic mice that expressed the murine 12/15-LO gene. Thus, we were able to use the mice as a novel tool to directly examine the role of 12/15-LO activity in mediating monocyte/endothelial interactions in vivo.

These mice had a 2-fold increase in 12/15-LO protein, which resulted in a modest, yet significant increase in 12/15-LO products in vivo (Fig. 4). We believe that the increased eicosanoid production we measured in vivo was a direct result of overexpression of the 12/15-LO gene in the mice and represents systemic production of these relevant eicosanoid products. However, we cannot rule out that these eicosanoid products were generated indirectly through activation of another pathway, such as through phospholipase A2 action on cellular phospholipids. Nevertheless, we found no obvious shunting of arachidonic acid to other eicosanoid pathways in the LOTG mice, suggesting that this production was specific to overexpression of the 12/15-LO enzyme.

Our study illustrates that 12/15-LO is a primary mediator of monocyte/endothelial interactions in the vessel wall. This is the first study that indicates that 12/15-LO products directly mediate monocyte adhesion to the endothelium through regulation of expression of key adhesion molecules in vivo. In LOTG mice, we found a significant increase in monocyte adhesion to aortic EC (Fig. 6). This increase in monocyte adhesion occurred even with basal unstimulated EC, suggesting that the LOTG EC were pre-activated to bind monocytes. There was little or no monocyte adhesion to aortic EC from LOKO mice (Fig. 9).

Direct inhibition of endothelial 12/15-LO using a 12/15-LO ribozyme construct or a pharmacological 12/15-LO inhibitor blocked monocyte adhesion to LOTG EC (Fig. 10). Finally,
addition of nanomolar levels of 12S-HETE (37, 39) to EC from LOKO mice significantly increased monocyte adhesion (Fig. 11). Taken together, these studies all illustrate the importance of endothelial 12/15-LO products in directly mediating monocyte/endothelial interactions in the vessel wall.

We chose to use nanomolar concentrations of 12S-HETE in some experiments based upon previously reported measurements of levels of 12S-HETE in human plasma and serum (40–42). In human plasma, levels of 12S-HETE have been reported to be in the range of 1–800 nM, depending on the method of quantification. In stimulated serum in vitro (through activation of platelets and leukocytes), this value increases to the low micromolar range (42). However, the exact concentration of active 12S-HETE is not known, as 12S-HETE can
readily bind to plasma proteins. Nevertheless, we used 100 nM to reflect a modest concentration of 12S-HETE that may be relevant to that found in the circulation.

The reasons for the observed “basal” activation of LOTG EC could be as follows: 1) an increase in adhesion molecule (VCAM-1, ICAM-1 expression on the EC surface, 2) an increase in CS-1 FN expression on the EC surface, and 3) and increase in endothelial chemokine production. We found a significant increase in ICAM-1 expression, but little or no increase in VCAM-1 expression, on endothelia of LOTG mice. We also found a significant decrease in ICAM-1 mRNA levels in LOKO mice. These data indicate that 12/15-LO somehow regulates ICAM-1 production. The mechanisms for this regulation are as yet unknown, but may relate to direct effects of eicosanoids on ICAM-1 mRNA expression. We found that blocking ICAM-1 in EC or its counterligand β3 integrin in monocytes significantly reduces monocyte adhesion to EC. Blocking ICAM-1 in EC reduced monocyte adhesion by 50%, and blocking β2 integrin reduced monocyte adhesion by ~60%. These data suggest that ICAM-1 is important in 12/15-LO-mediated monocyte adhesion. Use of a blocking antibody to VCAM-1 reduced adhesion to EC by ~30% (Fig. 13). However, blocking CS-1 FN on the EC surface using a peptide specific for the CS-1 FN-binding site on VLA-4 significantly reduced monocyte adhesion to EC by 60%. Blocking VLA-4 (by blocking binding sites for both VCAM-1 and CS-1 FN) reduced monocyte adhesion to LOTG EC by 80%. Additional studies will be needed to define the exact contributions of CS-1 FN versus VCAM-1 to mediating monocyte/endothelial adhesion in LOTG mice. We also need to examine the role of ICAM-2 in mediating monocyte adhesion because it is also a ligand for β3 integrins. Nevertheless, these studies reveal that ICAM-1/β2 integrin interactions as well as VCAM-1/CS-1 FN/α4 integrin interactions both play a role in 12/15-LO-mediated monocyte adhesion to EC in LOTG mice. We are currently in the process of examining the role of 12/15-LO activity in the modulation of endothelial chemokine production in LOTG mice, as this also contributes to monocyte/endothelial interactions.

We cannot yet rule out contributions of the platelet 12-LO enzyme to mediating monocyte/endothelial interactions in vivo. The platelet 12-LO pathway also generates 12S-HETE and is localized within circulating platelets. In our studies using isolated primary cultures of aortic EC from LOTG and LOKO mice, the platelet 12-LO pathway would not be a significant factor. However, in vivo, platelet 12-LO activity may be important for atherogenesis. Studies defining the role of platelet 12-LO in atherogenesis are currently underway in the laboratory.

In summary, overexpression of murine 12/15-LO in mice increases monocyte/endothelial interactions. These monocyte/endothelial interactions in LOTG mice are caused by endothelial CS-1 FN and VCAM-1 interactions with VLA-4 in monocytes and by endothelial ICAM-1 and β3 integrin interactions in monocytes. LOTG mice have significant up-regulation of endothelial ICAM-1 expression, suggesting that 12/15-LO regulates ICAM-1 production. LOTG mice develop spontaneous aortic fatty streak lesions on a rodent chow diet. Thus, the LOTG mouse is a new model that should provide novel mechanistic information regarding the role of 12/15-LO in mediating atherosclerotic vascular disease in vivo.

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