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Colocalization of 15-lipoxygenase mRNA and protein with epitopes of oxidized low density lipoprotein in macrophage-rich areas of atherosclerotic lesions

(*in situ* hybridization/immunocytochemistry/polymerase chain reaction/riboprobes/Watanabe heritable hyperlipidemic rabbit)

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ABSTRACT **Oxidation of low density lipoprotein (LDL) enhances its atherogenicity, and inhibition of such oxidation decreases the rate of progression of atherosclerotic lesions. The mechanism of LDL oxidation *in vivo* remains uncertain, but *in vitro* studies have suggested that cellular lipoxygenases may play a role by initiating lipid peroxidation in LDL. *In situ* hybridization studies using a 15-lipoxygenase riboprobe and immunostaining using antibodies against 15-lipoxygenase showed strongly positive reactivity largely confined to macrophage-rich areas of atherosclerotic lesions. Polymerase chain reaction with 15-lipoxygenase-specific oligonucleotides and restriction enzyme digestions of the amplified fragment were used to confirm the presence of 15-lipoxygenase message in the reverse-transcribed lesion mRNA. Immunostaining with antibodies reactive with oxidized LDL (but not with native LDL) indicated that the lipoxygenase colocalizes with epitopes of oxidized LDL, compatible with a role for macrophage lipoxygenase in the oxidation of LDL *in vivo*. Since oxidized LDL is chemotactic for blood monocytes, early lesions might progress at a markedly accelerated rate because of further recruitment of more monocytes which, in turn, would increase further the rate of oxidation of LDL. These data suggest that therapy targeted to block macrophage lipoxygenase activity might decrease the rate of development of atherosclerotic lesions.**

It is now well established that most of the lipid-loaded foam cells in early atherosclerotic lesions are derived from monocyte/macrophages that have entered the subendothelial space and taken up excess low density lipoprotein (LDL) (1). The uptake of native LDL by macrophages does not lead to generation of foam cells, but when LDL is chemically modified in one of several ways (e.g., by acetylation), it is taken up by an alternative receptor-mediated pathway that can lead to foam cell formation *in vitro* (2–5). One form of LDL recognized by the acetyl-LDL receptor is oxidatively modified LDL (5–8). This modification, initiated by lipid peroxidation, is accompanied by breakdown of the LDL apoprotein B and fragmentation of polyunsaturated fatty acids (5, 7). The fragmentation yields reactive aldehydes [such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE)] that conjugate to apoprotein B lysine residues (5, 9). Various cell types can modify LDL *in vitro* through oxidative processes (5, 6, 10, 11), and this modification is presumably analogous to the autooxidative modification of LDL in the absence of cells (5). Oxidized LDL is chemotactic for circulating monocytes (12), induces endothelial cells to release chemotactic substances (13), and is toxic to endothelial cells (14), all properties that further enhance its atherogenic potential (5).

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Chemically modified forms of LDL, such as acetylated LDL, have not been found *in vivo*, but there is increasing evidence for the occurrence of oxidized LDL *in vivo*: (*i*) immunocytochemical studies of rabbit arteries have shown that oxidized lipoproteins (and/or closely related proteins) are present in atherosclerotic lesions (15–18); (*ii*) LDL gently extracted from both human and rabbit lesions (but not from normal vessel wall) resembles oxidized LDL in its physical, biochemical, and immunological properties (19–21); (*iii*) human and rabbit serum contain autoantibodies against MDA- and 4-HNE-conjugated LDL (16, 22); and (*iv*) antioxidant therapy decreases the rate of progression of atherosclerotic lesions in Watanabe heritable hyperlipidemic (WHHL) rabbits (23, 24).

The mechanism of LDL oxidation *in vivo* has not been elucidated. In principle, it might result from the release of superoxide anion from cells, a direct action of membrane enzymes on LDL making contact with the cells, or the transfer of lipid peroxides generated within the cell membrane to LDL in the extracellular space. In any case, the initial "seeding" of LDL with a lipoperoxide would be followed by propagation, a process that may require metal ion catalysis. *In vitro* studies suggest that cellular lipoxygenases may play an important role (5, 25, 26). Thus, LDL treated with soybean lipoxygenase (LO) closely resembles LDL oxidized by incubation with cells (25). Moreover, several lipoxygenase inhibitors block cell-induced oxidation of LDL (26). Atherosclerotic lesions of rabbit aorta have been reported to show higher 15-LO activity than normal aorta (27, 28). However, those studies did not establish in which cell types the 15-LO resides or whether that expression relates to oxidation of LDL. The purpose of the present study was to determine whether LOs are present in atherosclerotic lesions, which cell types express LO mRNAs, and whether LOs colocalize with oxidized LDL in atherosclerotic lesions.

METHODS

Studies were done utilizing the WHHL rabbit, a strain deficient in functional LDL receptors and therefore showing marked elevation of LDL and spontaneous atherosclerosis (29). Grossly visible lesions were dissected from the aortic arch and the thoracic aorta of six WHHL rabbits (1–2 years old). Animals were anesthetized with intramuscular ketamine (35 mg/kg) and xylazine (5 mg/kg), exsanguinated, and

Abbreviations: PCR, polymerase chain reaction; LO, lipoxygenase; 4-HNE, 4-hydroxynonenal; MDA, malondialdehyde; nt, nucleotide; LDL, low density lipoprotein; WHHL rabbit, Watanabe heritable hyperlipidemic rabbit.

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perfused 3 min with physiologic saline containing 1 mM EDTA and 50 mM butylated hydroxytoluene (BHT) followed by perfusion fixation for 5 min with formal/sucrose (4% paraformaldehyde/5% sucrose/1 mM EDTA/50 μM BHT, pH 7.4). Aortic tissue was immediately removed and immersion fixed in formal/sucrose containing antioxidants for another 6 hr. Serial paraffin-embedded sections (10 μm) were used for *in situ* hybridization and immunocytochemistry. Unfixed aortic lesions were dissected from two other WHHL rabbits of the same age, homogenized, and used for Western blotting analysis to confirm the presence of 15-LO enzyme protein in the lesions. Two additional WHHL rabbits were used for the isolation of total RNA from dissected atherosclerotic lesions. All animal studies were approved by the Animal Care and Use Committee of the University of California, San Diego.

Human 15-LO cDNA (30), human 5-LO cDNA (31), and human retinoic acid receptor cDNA (32) were subcloned in plasmid vectors as follows, using standard techniques (33). Human 15-LO cDNA: an *Eco*RI fragment containing nucleotides 1 to 2716 was subcloned in pBluescript (Stratagene). Human 5-LO: an *Eco*RI–*Bcl* I fragment containing nucleotides −34 to 2185 was subcloned in pTZ19R (Pharmacia). Human retinoic acid receptor cDNA: a *Bam*HI–*Pst* I fragment containing nucleotides 1 to 547 was subcloned in pGEM4Z (Promega). Because of the high sequence identity between LO enzymes across species (34, 35), the human 15-LO and 5-LO cDNA probes would be expected to recognize corresponding rabbit sequences. This was confirmed by using Northern blotting techniques with rabbit reticulocyte mRNA (15-LO) and rabbit leukocyte mRNA (5-LO). Human retinoic acid receptor cDNA was used as an irrelevant probe, and this did not hybridize to the rabbit tissues. Antisense and sense riboprobes were synthesized by using Sp6, T7, or T3 RNA polymerases with uridine 5′-[α-[^{35}S]thio]triphosphate (1200 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq) (36). The specific activity of the probes varied from 250 to 300 Ci/mmol. Reagents used for riboprobe synthesis were supplied by Promega except that T3 RNA polymerase was from Stratagene. After the synthesis, cDNA templates were digested with 2 units of RNase-free DNase (Promega) for 30 min at 37°C and unincorporated isotope was removed by spin columns (Nu-Clean R50; International Biotechnologies). Samples were then extracted with phenol/chloroform and chloroform, precipitated with ethanol in the presence of excess yeast tRNA, and stored as ethanol precipitates at −70°C until used for hybridization studies. In most experiments probe sizes were reduced by alkaline hydrolysis (37) in 0.1 M sodium carbonate buffer (pH 10.2) to an average length of 250 nucleotides (nt). Probe sizes were confirmed by polyacrylamide or agarose/formaldehyde gel electrophoresis and their specificity was tested on Northern blot analysis.

Tissue sections for *in situ* hybridization were deparaffinized, digested with proteinase K (1 μg/ml, 10 min at 37°C; Boehringer Mannheim), and acetylated (0.25% acetic anhydride in 0.1 M triethanolamine, 10 min, 20°C) (38, 39). Slides were washed twice in 2× SSC (1× SSC = 150 mM NaCl/15 mM sodium citrate, pH 7.0), dehydrated, and dried under reduced pressure. Each hybridization experiment was done from a set of serial sections using an antisense probe and a corresponding nonhybridizing sense probe; 45 μl of each probe (3–5 × 10^6 cpm/ml in hybridization solution) was added to the slides and they were hybridized at 50–52°C for 14 hr. The slides were then washed four times (15 min each) in 4× SSC, digested with RNase A (20 μg/ml; Sigma) for 30 min at 37°C, and washed again at room temperature with 2× SSC, 1× SSC, and 0.5× SSC for 15 min (two times each). The final wash was with 0.1× SSC at 60°C for 60 min. Each washing solution after the RNase A step contained 1 mM dithiothreitol and 1 mM EDTA. Slides were then dried, dipped in photographic emulsion (NTB-2; Eastman Kodak), and developed after 2–6 weeks of exposure time. *In situ* hybridization solution contains 50% (vol/vol) formamide (Fluka), 2× SSC, 20 mM Tris·HCl, pH 7.4, 1× Denhardt's solution, 1 mM EDTA, 10% (wt/vol) dextran sulfate (Pharmacia), 1 mM dithiothreitol, and yeast tRNA (Boehringer Mannheim) at 0.5 mg/ml [1× Denhardt's solution is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin (all from Sigma)].

Serial sections were used for immunocytochemistry using previously described antibodies: mouse monoclonal antibodies against macrophages (RAM-11) (40), smooth muscle cells (HHF-35) (41), and LDL apoprotein B (MB47) (42); polyclonal guinea pig antisera against MDA- and 4-HNE-modified LDL (MAL-2 and HNE-6, respectively) (16, 22); and Sepharose G-purified rabbit (43) and goat antisera against human recombinant 15-LO. In addition we prepared a guinea pig antiserum against purified rabbit reticulocyte 15-LO. An avidin–biotin–horseradish peroxidase system was used for the immunostaining (Vector Laboratories). After the immunostaining the slides were counterstained with methyl green.

Poly(A)-selected mRNA was isolated (33) from four atherosclerotic lesions (total wet weight, 200 mg) after microscale extraction with guanidine thiocyanate and CsCl centrifugation (44). mRNA was reverse-transcribed for 1 hr at 42°C (33) by using 40 units of avian myeloblastosis virus reverse transcriptase (Seikagaku Kogya, Tokyo) and 0.2 μg of oligo(dT) as a primer. One-tenth of the resulting cDNA was amplified with *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin–Elmer/Cetus) during 40 cycles of polymerase chain reaction (PCR) (45) on a thermocycler (Ericomp, San Diego, CA). Each cycle included denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and primer extension at 72°C for 2 min. PCR reaction mixtures contained 200 μM final concentration of each dNTP (including [α-^{32}P]dCTP from New England Nuclear at 1 μCi/50 μl), each primer at 1.0 μM (see below), 2.5 units of *Taq* polymerase, 1× PCR buffer (10× PCR buffer contains 100 mM Tris·HCl at pH 8.3, 15 mM $MgCl_2$, 500 mM KCl, and 0.01% gelatin), and H_2O to a final volume of 50 μl. In all experiments, the presence of contaminants was checked by control reactions in which PCR was carried out on samples in which (*i*) reverse transcriptase was omitted and (*ii*) cDNA template was omitted. An aliquot of each amplification reaction mixture was subjected to restriction enzyme digestions (all enzymes from Bethesda Research Laboratories) and/or electrophoresis on 2.5% agarose gels or 5% polyacrylamide gels (33).

Oligonucleotides used for PCR were 24-mers of the following sequences (Stratagene): sense primer 5′-GGY-TTG-CCA-CTT-TGT-CAC-CAT-GTG-3′ (Y = C or T); antisense primer 5′-TCC-GGA-YCT-CAA-TTT-CCT-TAT-CCA-3′. These oligonucleotides are predicted to amplify a 349-base-pair (bp) fragment (nucleotides 1602–1951) from rabbit reticulocyte 15-LO sequence (35).

RESULTS

Hybridization of the sections with 15-LO antisense probe showed mRNA expression in the subendothelial and "shoulder" regions (lateral margins) of both very early and more advanced atherosclerotic lesions. Weak hybridization was also detected in the endothelium in some, but not all, areas. Cells in the deeper portions of intima did not express this LO mRNA. An example of the mRNA expression in a WHHL rabbit atherosclerotic lesion is shown in Fig. 1*A*. Neither the 15-LO sense probe (Fig. 1*B*) nor the 5-LO antisense probe (Fig. 1*C*) hybridized to the sections. The 15-LO signal was abolished by pretreatment of the sections with RNase A; hybridization with an irrelevant antisense probe (human retinoic acid receptor) gave no signal (data not shown). In

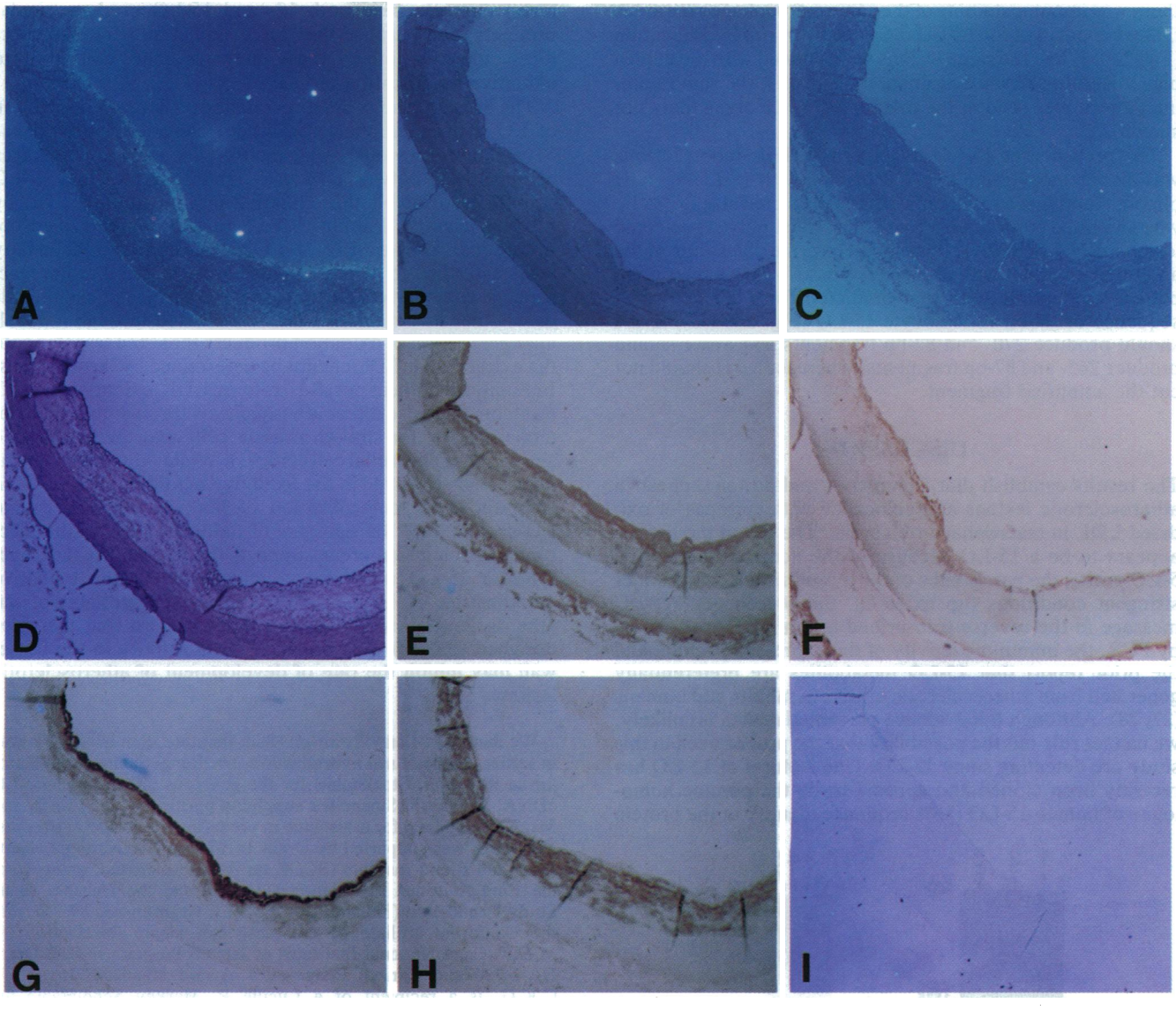

FIG. 1. 15-LO mRNA expression and 15-LO protein in the WHHL rabbit atherosclerotic lesions colocalizes with macrophages and with oxidized LDL epitopes. *In situ* hybridization was carried out with ^{35}S-labeled riboprobes in formal/sucrose-fixed, paraffin-embedded sections (10 μm) from upper thoracic aorta of a WHHL rabbit. (*A*) 15-LO antisense probe. (*B*) 15-LO nonhybridizing sense probe. (*C*) 5-LO antisense probe. (Exposure time in *A–C* was 3 weeks.) (*D*) A serial section stained with hematoxylin and eosin. (*E–I*) Immunocytochemistry of the serial sections (avidin–biotin–horseradish peroxidase system). (*E*) Antibody against MDA-modified LDL (MAL-2; 1:500 dilution). (*F*) Antibody against human 15-LO protein that cross-reacts with rabbit 15-LO (goat antiserum, 1:500 dilution). (*G*) Antibody specific for macrophages (RAM-11; 1:1000 dilution). (*H*) Antibody against native LDL apoprotein B (MB47; 1:500 dilution). (*I*) Nonimmune control for the immunostaining. (Methyl green counter stain in *E–I*. Photographs in *A–C* were taken with polarized light epiluminescence; photographs in *D–I* were taken with bright-field illumination. All 24×.)

contrast to the strong signal in lesioned areas, only a weak signal, if any, was seen in normal regions of the aorta when the 15-LO antisense probe was used. No detectable expression of 5-LO mRNA was seen in normal vessel wall or in the endothelium (data not shown).

Immunostaining using a macrophage-specific monoclonal antibody (RAM-11) revealed that the cells reacting with the 15-LO probe were macrophages (Fig. 1*G*). Immunostaining with an antiserum against MDA-modified LDL (Fig. 1*E*) was localized to the same areas as 15-LO mRNA and macrophages. Staining with antisera against 4-HNE-LDL gave identical results (data not shown). Thus, MDA- and 4-HNE-modified LDL and/or related modified proteins were present in these areas. A monoclonal antibody against apoprotein B (apo B) (MB47) detected no apo B in the macrophage-rich areas, whereas it was reactive in the deeper portions of intima in areas that contained no macrophages (Fig. 1*H*). Previous studies using MB47 and a polyvalent antiserum against apo B have given similar results—i.e., there was very little reactive apo B in macrophage-rich areas of the lesions (18).

To test for the expression of 15-LO protein in atherosclerotic lesions, immunostaining and immunoblots with 15-LO antibodies were performed. Sonicated extracts of WHHL aortic lesions were subjected to Western blotting, using a previously described rabbit anti-human recombinant 15-LO antibody that cross-reacts with rabbit 15-LO but does not recognize either human 5-LO (43) or human platelet 12-LO (E.S., unpublished observations). A major band (70,000 Da) was immunostained that comigrated with authentic rabbit reticulocyte 15-LO (data not shown). To stain the WHHL rabbit lesions, a goat antiserum was raised against human recombinant 15-LO and shown to cross-react with rabbit reticulocyte 15-LO. When this antibody was applied to the same lesions used for the *in situ* studies, it was observed that

there was immunostaining of the same macrophage-rich areas that were positive for 15-LO message by *in situ* hybridization (Fig. 1*F*). When the same lesions were stained with the guinea pig antiserum prepared against rabbit 15-LO, the same staining pattern was seen in the macrophage-rich areas (data not shown).

The presence of 15-LO mRNA in WHHL rabbit lesions was also demonstrated by PCR primed with 15-LO-specific oligonucleotides: a fragment with the predicted size of 349 bp was amplified from the reverse-transcribed poly(A)-selected lesion mRNA (Fig. 2*A*). Restriction enzyme digestions of the amplified fragment with three enzymes also gave a restriction pattern compatible with that predicted from the published sequence of rabbit reticulocyte 15-LO (Fig. 2*B*) (35): *Ava* I should produce 270- and 82-bp fragments and *Sca* I should produce 265- and 87-bp fragments, whereas *Pvu* II should not cut the amplified fragment.

DISCUSSION

The results establish that LO mRNA and protein appear in atherosclerotic lesions and colocalize with epitopes of oxidized LDL in macrophage-rich areas. The LO in the lesions appears to be a 15-LO because of the nonreactivity of the 5-LO probe, the persistence of the hybridization signal under stringent conditions (up to 60°C), the presence of 15-LO message in the reverse-transcribed lesion mRNA as shown by PCR, the immunoreactivity of the expressed protein, and the prior report that 15-LO metabolites are preferentially generated from atherosclerotic lesions in rabbits and humans (27, 28). Although the evidence presented makes it unlikely, we cannot rule out the possibility that the probes used in this study are detecting some 12-LO. One isoform of 12-LO has recently been cloned and appears to be the porcine homologue of human 15-LO (86% sequence identity at the protein level) (46). Because both 12- and 15-LO produce similar products, and because they have similar substrate specificities, either or both of these enzymes could contribute to oxidative modification of LDL.

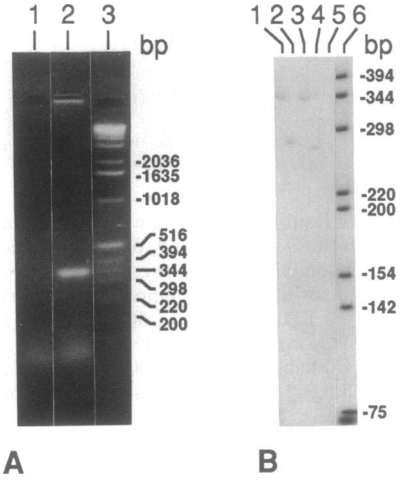

FIG. 2. Demonstration of 15-LO expression in WHHL rabbit atherosclerotic lesions by using PCR. (*A*) Amplification (40 cycles) of poly(A)-selected, reverse-transcribed lesion mRNA primed with 15-LO-specific 24-mer oligonucleotides. Lane 1, control tube amplified without cDNA template; lane 2, lesion cDNA giving a predicted 349-bp product; lane 3, molecular weight markers. Samples were run on a 2.5% agarose gel with ethidium bromide staining. (*B*) Restriction enzyme digestion of the amplified 349-bp fragment labeled with [α-^{32}P]CTP during PCR. Lane 1, amplified fragment; lane 2, digestion of the fragment with *Ava* I; lane 3, digestion with *Pvu* II; lane 4, digestion with *Sca* I; lane 5, control tube amplified without cDNA template; lane 6, molecular weight markers. The sizes of the fragments observed in lanes 2 and 4 are as predicted from the published sequence of the rabbit reticulocyte 15-LO, whereas in accordance with the sequence information *Pvu* II does not cut the amplified fragment. Samples were run on a 5% polyacrylamide gel and reaction products were visualized after a 24-hr exposure on Kodak XAR film.

The present results demonstrate the striking colocalization of LO with macrophages in atherosclerotic lesions. It has been shown previously that mouse peritoneal macrophages exhibit LO activity (47). *In vitro*, inhibitors that block 15-LO activity also inhibit the oxidative modification of LDL by macrophages (S. Rankin, S.P., and D.S., unpublished observations). Unlike 5-LO, 15-LO has the ability to act on fatty acids esterified to phospholipids (48, 49). Whether the macrophage enzyme acts directly on LDL surface phospholipids or whether its action on LDL is mediated through intracellular generation of oxidation products that then transfer onto the extracellular LDL cannot be determined by these studies. In addition to their possible role in LDL oxidation, 15-LO products may also affect atherogenesis through their chemotactic activity for smooth muscle cells and their mitogenic activity for endothelial cells (50, 51). While other mechanisms may also be involved in the oxidative modification of LDL *in vivo*, the close colocalization of both 15-LO mRNA and LO-protein with the epitopes of oxidized LDL in the macrophage-rich lesion areas supports the hypothesis that macrophage 15-LO may play a major role in the oxidative modification of LDL and thus in the progression of the atherosclerotic lesion. These studies suggest that therapies designed to block macrophage 15-LO activity in the vessel wall may inhibit the rate of development of atherosclerotic lesions.

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