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Macrophages and smooth muscle cells express lipoprotein lipase in human and rabbit atherosclerotic lesions

(*in situ* hybridization/immunocytochemistry/riboprobes/mRNA/foam cells)

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ABSTRACT **Lipoprotein lipase (LPL; EC 3.1.1.34) may promote atherogenesis by producing remnant lipoproteins on the endothelial surface and by acting on lipoproteins in the artery wall. *In vitro*, smooth muscle cells and macrophages synthesize LPL, but in human carotid lesions only a few smooth muscle cells were reported to contain LPL protein. Endothelial cells do not synthesize LPL *in vitro*, but in normal arteries intense immunostaining for LPL is present on the endothelium. We used Northern blot analysis, *in situ* hybridization, and immunocytochemistry of human and rabbit arteries to determine cellular distribution and the site of the synthesis of LPL in atherosclerotic lesions. Northern blot analysis showed that LPL mRNA was detectable in macrophage-derived foam cells isolated from arterial lesions of "ballooned" cholesterol-fed rabbits. *In situ* hybridization studies of atherosclerotic lesions with an antisense riboprobe showed a strong hybridization signal for LPL mRNA in some, but not all, lesion macrophages, which were mostly located in the subendothelial and edge areas of the lesions. Also, some smooth muscle cells in lesion areas also expressed LPL mRNA. Immunocytochemistry of frozen sections of rabbit lesions with a monoclonal antibody to human milk LPL showed intense staining for LPL protein in macrophage-rich intimal lesions. The results suggest that lesion macrophages and macrophage-derived foam cells express LPL mRNA and protein. Some smooth muscle cells in the lesion areas also synthesize LPL. These data are consistent with an important role for LPL in atherogenesis.**

Lipoprotein lipase (LPL; EC 3.1.1.34) is the enzyme primarily responsible for the hydrolysis of lipoprotein triglycerides at the capillary endothelium with the subsequent uptake of free fatty acids and monoglycerides by parenchymal cells (1). LPL is produced in a number of tissues, including brown and white adipose tissue, skeletal and heart muscle, mammary gland and brain, and macrophages (2–7). In all tissues studied LPL is synthesized by parenchymal cells and is then released for transfer to the endothelial surface where it is bound to heparan sulfate proteoglycans. Endothelial cells do not themselves synthesize LPL (1, 6).

It has been suggested by Zilversmit (8) that LPL may promote atherosclerosis by producing remnant lipoproteins on the endothelial surface. Remnant lipoproteins are potentially more atherogenic than native LDL since they are taken up more avidly by macrophages (9). In addition, LPL activity is probably present *within* the artery wall itself (10, 11) and particles resembling remnant lipoproteins have been isolated from human arterial wall (12). In addition to the localized production of remnant particles within the artery wall, increased expression of LPL within atherosclerotic lesions might lead to an enhanced transport of LPL to the endothelial surface overlying the lesions, generating remnant lipoproteins that could then enter the lesion area. However, little explicit information is available about the expression of LPL mRNA and protein in atherosclerotic lesions *in vivo* (13).

The purpose of the present study was to determine, first, whether LPL mRNA and protein are present in human and rabbit atherosclerotic lesions, and second, which cell types are responsible for its expression.

METHODS

Tissue Samples. Human samples were obtained from two organ donors for renal transplant (abdominal aorta; ages 28 and 33 years) and from two autopsy donors (thoracic aorta; ages 37 and 41 years; postmortem times, 3.5 hr and 5 hr, respectively). Samples were immersion fixed overnight at 4°C in formal/sucrose (4% paraformaldehyde/5% sucrose/1 mM EDTA/50 μM butylated hydroxytoluene, pH 7.4) and embedded in paraffin (14, 15).

Six Watanabe heritable hyperlipidemic (WHHL) rabbits (1–2 years old), three control New Zealand White rabbits, and six cholesterol-fed New Zealand White rabbits (1% cholesterol diet for 10 weeks) were anesthetized with intramuscular ketamine (35 mg/kg) and xylazine (5 mg/kg) and perfused briefly with physiologic saline containing 1 mM EDTA and 50 μM butylated hydroxytoluene. Aortic tissue was then immediately removed, embedded in OCT compound (Miles Scientific), frozen in isopentane/liquid N_2, and kept at −70°C until analyzed (14, 15).

All human and animal studies were approved by the Human Subjects Committee and the Animal Subjects Committee of the University of California, San Diego.

Isolation of Arterial Foam Cells. Macrophage-derived foam cells were isolated as described (16). Briefly, atherosclerotic lesions were induced in 14 New Zealand White rabbits by balloon deendothelialization of the aorta; this was followed by feeding of a 2% cholesterol diet for 10–11 weeks to induce hypercholesterolemia. Foam cells were isolated from the dissected lesions using collagenase and elastase digestions and purified by a discontinuous metrizamide gradient centrifugation as described (16). Isolated foam cell preparations contained essentially no contaminating smooth muscle cells (16). Alveolar macrophages were isolated simultaneously from the same animals using bronchoalveolar lavage with phosphate-buffered saline. As a further control, alveolar macrophages were treated with the same metrizamide and collagenase/elastase solutions used for the isolation of arterial foam cells. All solutions and reagents were free of

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Abbreviations: LPL, lipoprotein lipase; PMA, phorbol 12-myristate 13-acetate; VLDL, very low density lipoprotein; WHHL, Watanabe heritable hyperlipidemic.

detectable endotoxin contamination as assayed by the *Limulus* assay (Whittaker Bioproducts).

Probes. A 2.4-kilobase (kb) *Sal* I fragment of human LPL cDNA (17) and a 1.4-kb *Eco*RI fragment of mouse LPL cDNA (18) were subcloned (19) into pGem2 (Promega). Because of the high homology between human and mouse LPL sequences, the mouse and human probes were considered suitable for studies with rabbit tissues; this was confirmed by preliminary Northern blotting analysis, which demonstrated that rabbit adipose tissue RNA was positive for LPL. A human retinoic acid receptor cDNA (0.55-kb *Bam*HI–*Pst* I fragment) was used in *in situ* hybridization studies as an irrelevant control probe (14) that did not hybridize to human or rabbit arterial tissue.

Antisense and sense riboprobes were synthesized using Sp6 or T7 RNA polymerases with UTP[^{35}S] (1200 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) as a labeled nucleotide (14, 20). For *in situ* hybridization studies, probe sizes were reduced by alkaline hydrolysis to an average length of 250 nucleotides (14, 15). Probe sizes were confirmed by polyacrylamide gel electrophoresis and probe specificity was tested on Northern blot analysis (19).

***In Situ* Hybridization.** *In situ* hybridizations were done as described (14, 15). All studies were done on a set of serial sections (10 μm) using an antisense probe, a corresponding nonhybridizing sense probe, and an irrelevant nonhybridizing probe. Tissue sections were deparaffinized, subjected to pretreatment with proteinase K (1 μg/ml, 15 min at 37°C; Boehringer Mannheim), and then acetylated (0.25% acetic anhydride in 0.1 M triethanolamine, 10 min at 20°C). Sections were then washed twice with 2× SSC (1× SSC = 150 mM NaCl/15 mM sodium citrate, pH 7.0), dehydrated, and dried *in vacuo*. Slides were hybridized for 14 hr at 50–52°C in a hybridization solution (see below) containing 3–6 × 10^6 cpm/ml of each probe (14, 15). In some experiments tissue sections were pretreated with RNase A, which abolished the signal.

Posthybridization treatment involved the following. Slides were washed three times in 4× SSC (15 min each) and digested with RNase A (20 μg/ml; Boehringer Mannheim) for 30 min at 37°C. The slides were then washed for 15 min at 20°C with 2× SSC, 1× SSC, and 0.5× SSC (two times each). The final wash was with 0.1× SSC at 60°C (human tissue) or at 55°C (rabbit tissue) for 60 min. Slides were then dehydrated, dipped in photographic emulsion (NTB-2; Eastman Kodak), and developed after 4–12 weeks' exposure time (14, 15). After the development, slides were counterstained with hematoxylin/eosin. The *in situ* hybridization solution contains 50% formamide (Fluka), 2× SSC, 20 mM Tris (pH 7.4), 1× Denhardt's solution (0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin, all from Sigma), 1 mM EDTA, 10% dextran sulfate (Pharmacia), 1 mM dithiothreitol, and 0.5 mg of yeast tRNA per ml (Boehringer Mannheim).

Immunocytochemistry. Paraffin-embedded serial sections (10 μm) from human aortas were immunostained with mouse monoclonal antibodies against smooth muscle cells (HHF-35) (21) and human macrophages (HAM-56) (22). Frozen sections (10 μm) from rabbit aortas were immunostained using mouse monoclonal antibodies against human milk LPL (ascites fluid) (23) and rabbit macrophages (RAM-11) (24). Preliminary studies demonstrated that the monoclonal antibody to human LPL reacted with rabbit adipose tissue. However, with this antibody LPL immunostaining was detectable only in frozen sections, and fixation dramatically decreased immunoreactivity. The avidin/biotin/horseradish peroxidase or alkaline phosphatase systems were used for the signal detection (Vector Laboratories). After immunocytochemistry the slides were counterstained with methyl green.

Northern Blotting Analysis. Total RNA was isolated from freshly prepared arterial foam cells and alveolar macrophages (10–30 × 10^6 cells) using guanidinium isothiocyanate extraction and CsCl centrifugation (19). As an additional control for the inducible expression of LPL mRNA, total RNA was isolated from an equal number of human monocytic cells (THP-1; American Type Culture Collection) cultured in RPMI 1640 medium supplemented with 10% fetal calf serum after 0, 3, and 12 hr of stimulation with phorbol 12-myristate 13-acetate (PMA; 0.1 μM; Sigma) (25). Ethanol-precipitated RNA was electrophoresed on 1% agarose/formaldehyde gels and transblotted to nylon membranes (Nytran, Schleicher & Schuell) (19). Human or mouse LPL cDNA was labeled with [^{32}P]CTP (3000 Ci/mmol; ICN) using random priming (BRL), and the membranes were hybridized (1 × 10^6 cpm/ml) overnight at 42°C according to manufacturer's instructions. The final wash was with 0.25× SSC/0.2% SDS at 42°C for 30 min. Membranes were then dried and the signal was detected using autoradiography (Kodak XAR-5 film).

RESULTS

LPL mRNA was detected in freshly obtained macrophage-derived foam cells isolated from rabbit atherosclerotic lesions induced by balloon deendothelialization and diet-induced hypercholesterolemia (Fig. 1). Alveolar macrophages were simultaneously isolated from the same animals using the same protocol and also found to be positive for LPL mRNA (Fig. 1). To rule out a possible induction of LPL mRNA in foam cells and alveolar macrophages during the isolation process, cultured THP-1 cells were used as an additional control. Since LPL mRNA expression is induced in THP-1 cells only after 6 hr of incubation with PMA (25), THP-1 cells were stimulated with 0.1 μM PMA for 0, 3, and 12 hr and analyzed for the presence of LPL mRNA; no detectable LPL mRNA was present after 0 hr (data not shown) or 3 hr of stimulation. The 3-hr time point corresponds to the time

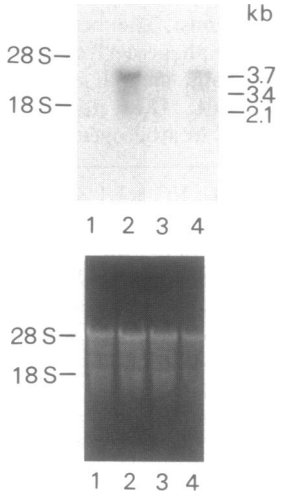

FIG. 1. Northern blot analysis of LPL mRNA in rabbit lesion macrophage-derived foam cells, alveolar macrophages, and PMA-stimulated THP-1 cells. Total RNA was isolated and subjected to Northern blot analysis (25 μg of total RNA per lane). (*Upper*) Northern blot with a [^{32}P]CTP-labeled mouse LPL cDNA probe. Lane 1, THP-1 cells stimulated for only 3 hr with 0.1 μM PMA; lane 2, THP-1 cells stimulated 12 hr with 0.1 μM PMA; lane 3, foam cells isolated from ballooned aorta of cholesterol-fed rabbits; lane 4, alveolar macrophages isolated simultaneously from the ballooned cholesterol-fed animals. The size of the major LPL mRNA species is 3.7 kb. Occasionally two smaller mRNA bands of 3.4 kb and 2.1 kb were detectable. (*Lower*) Ethidium bromide staining of the RNA gel. Lanes 1–4 correspond to those in *Upper*. Positions of the 28S and 18S rRNA subunits are indicated.

required for the isolation of foam cells. A strong signal was detected after 12 hr of stimulation with PMA (Fig. 1). Thus, it is unlikely that the expression of LPL mRNA in isolated foam cells and alveolar macrophages is due to artifactual activation during the 3-hr isolation procedure.

Hybridization of human atherosclerotic lesions with a human LPL antisense riboprobe demonstrated distinctive LPL mRNA expression in some lesion macrophages and smooth muscle cells (Fig. 2). An example of the macrophage expression of LPL mRNA in a human fatty streak is seen in Fig. 2*A*. No hybridization was seen with the corresponding sense probe in an adjacent section (Fig. 2*B*) nor with an irrelevant antisense riboprobe (human retinoic acid receptor; data not shown). Immunostaining of adjacent serial sections showed that the cells positive for LPL mRNA were macrophages (Fig. 2*C*). Fig. 2*D* shows LPL mRNA expression in another fatty streak lesion from the same donor. Only a minority of macrophages was positive and we estimate that only 10–20% of the lesion macrophages expressed LPL mRNA.

An example of the expression of LPL mRNA by smooth muscle cells in human lesions is seen in Fig. 2*E*; cells located in the inner intima expressed LPL mRNA. Immunostaining of serial sections revealed that most of the positive cells were probably smooth muscle cells (Fig. 2*F*), particularly since the area positive for LPL mRNA in this tissue section did not contain a significant number of macrophages (Fig. 2*G*). However, we estimate that overall <15% of the lesion smooth muscle cells were positive for LPL mRNA. For all *in situ* hybridization experiments, pretreatment of the sections with RNase A abolished the signal (data not shown) and nonimmune controls for the immunohistochemical studies were negative.

We could not detect an *in situ* hybridization signal in endothelial cells. We did observe a slightly increased grain count (compared to background) over smooth muscle cells in normal media. However, it is unclear whether this truly represents a low level of constitutive expression of LPL mRNA or just increased background signal (data not shown).

In situ hybridization of rabbit sections with the human LPL antisense riboprobe also showed mRNA expression in macrophage-rich lesion areas (data not shown). Immunostaining of the frozen sections of lesions from WHHL and cholesterol-fed New Zealand White rabbits (using a monoclonal antibody

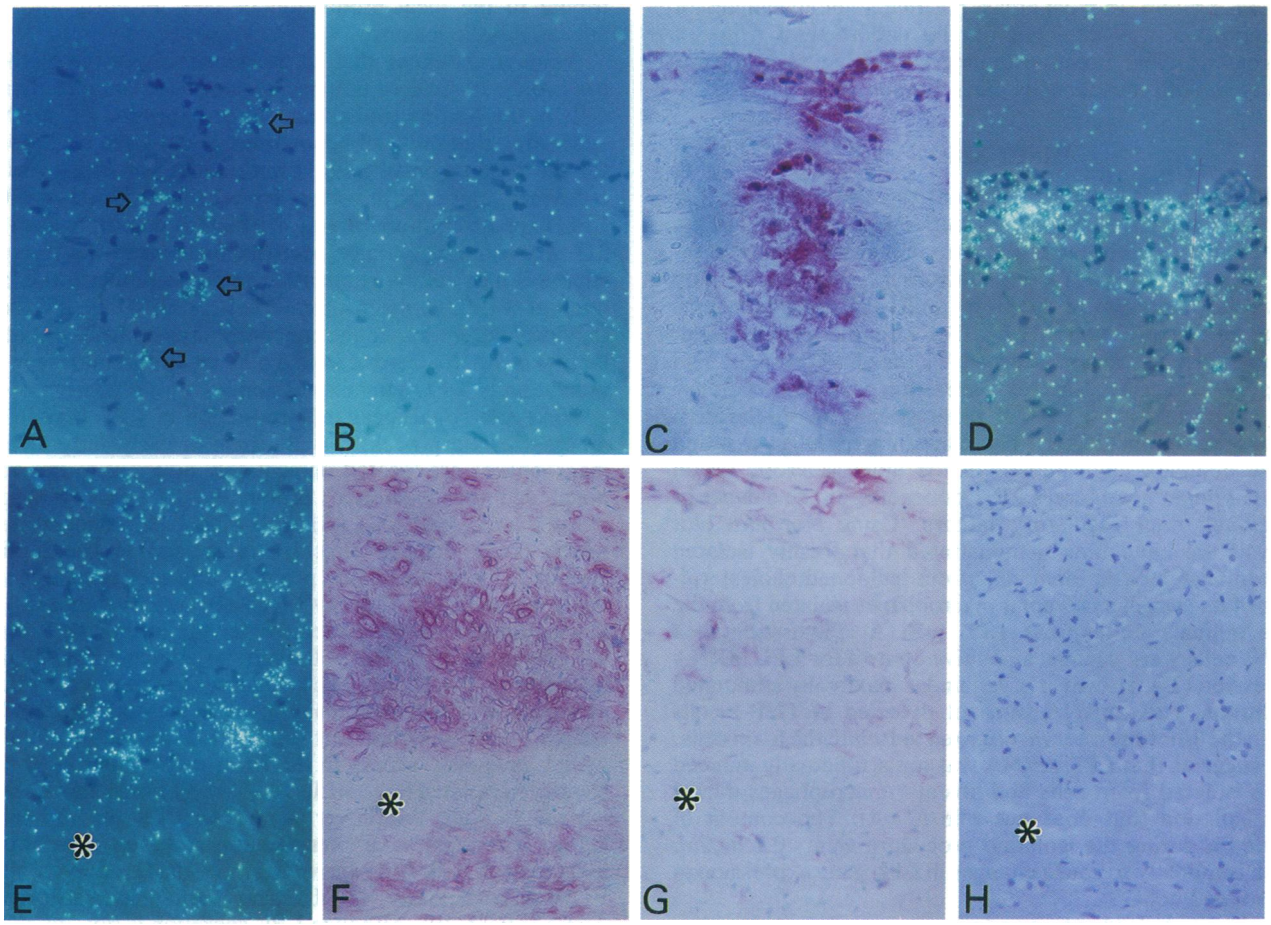

FIG. 2. LPL mRNA expression in human atherosclerotic lesions. Tissues were fixed in formal/sucrose and paraffin-embedded. (*A–C*) Serial sections through a cap region of a fibrous plague (abdominal aorta, 41-yr-old male; postmortem time, 3.5 hr). (*A*) *In situ* hybridization with a human LPL antisense probe. Open arrows indicate the positive cells. (*B*) Control *in situ* hybridization of adjacent section with the corresponding nonhybridizing sense probe. (*C*) Immunostaining (avidin/biotin/alkaline phosphatase system) for human macrophages (HAM-56; 1:1000 dilution). (*D*) Another macrophage-rich fatty streak (same tissue donor). *In situ* hybridization with the human LPL antisense probe. (*E–H*) Serial sections through a fibrous plaque (same tissue donor; inner intimal layer adjacent to a fibrous streak marked with asterisk). (*E*) *In situ* hybridization with the human LPL antisense probe. (*F*) Immunostaining for smooth muscle cells (HHF-35; dilution 1:1000). (*G*) Immunostaining of adjacent section for human macrophages (HAM-56; 1:1000 dilution). (*H*) Hematoxylin/eosin staining. Nonimmune controls for the immunostainings were negative (data not shown). Exposure time for *in situ* hybridization autoradiography was 12 weeks. Hematoxylin/eosin counterstain was used in *in situ* hybridization sections; methyl green counterstain was used in other sections. The *in situ* hybridization photographs were taken using polarized light epiluminescence; other photographs were taken using bright-field illumination. (*A–E*, ×200; *F–H*, ×100.)

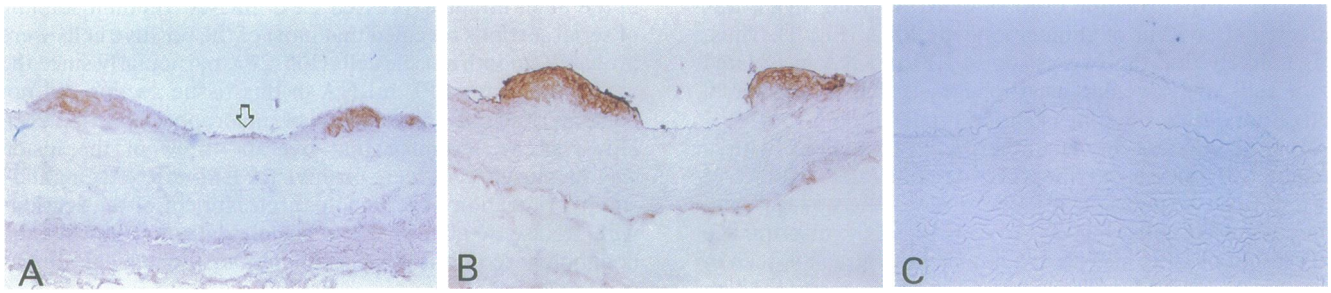

FIG. 3. Immunostaining for LPL protein in rabbit macrophage-rich fatty streaks (serial frozen sections, thoracic aorta, 1-yr-old WHHL rabbit). (*A*) Monoclonal antibody specific for human milk LPL that crossreacts with rabbit LPL (ascites fluid; 1:25 dilution); both lesions stain positive for LPL. An arrow indicates positive staining on the endothelium between the two lesions. (*B*) Adjacent section stained with antibody specific for rabbit macrophages (RAM-11; 1:1000 dilution). (*C*) Nonimmune control for the immunostaining. The avidin/biotin/horseradish peroxidase system and methyl green counterstain were used. (*A* and *B*, ×47; *C*, ×94.)

against human milk LPL that crossreacts with rabbit LPL) showed the presence of LPL protein (Fig. 3*A*). The immunostaining pattern using a macrophage-specific monoclonal antibody suggested that the cells responsible for the expression of LPL protein were macrophages (Fig. 3*B*), although, again, only a small fraction of the macrophages was positive for LPL protein. In most areas, positive immunostaining for LPL protein was detected on the endothelium (Fig. 3), whereas no clear immunostaining was detected in normal medial smooth muscle cells (data not shown).

DISCUSSION

Studies from this laboratory demonstrated that macrophages in culture constitutively secrete active LPL into the culture medium (2, 3). Subsequently, many laboratories have demonstrated that many factors, including cytokines already shown to be present in the artery wall, can regulate macrophage LPL expression *in vitro*. For example, retinoic acid, dexamethasone, tumor necrosis factor, interferon γ, and lipopolysaccharide suppress secretion, whereas colony-stimulating factors, PMA, or other stimuli of proliferation increase secretion (26–31). Therefore, macrophages exposed to the complex environment of the atherosclerotic lesion might or might not express LPL, depending on the balance of these and other regulatory factors. Using Northern blot analysis we have now demonstrated that freshly isolated macrophage-derived foam cells from ballooned cholesterol-fed rabbits as well as alveolar macrophages isolated from the same animals do express LPL mRNA. PMA-stimulated THP-1 cells were used as a positive control for LPL mRNA expression (25). However, even under maximally stimulated conditions, LPL mRNA was not detected in THP-1 cells within the 3-hr time interval required to isolate the foam cells. This suggests that LPL mRNA was not artifactually induced in the isolated foam cells and alveolar macrophages during their isolation (but does not rule it out). The content of endotoxin during the isolation procedure was <0.1 ng/ml, which is unlikely to cause changes in LPL gene expression in the isolated cells.

The *in situ* hybridization and immunocytochemical studies definitively demonstrate that human and rabbit lesions contain LPL mRNA and protein, in lesion macrophages and smooth muscle cells. The results are consistent with *in vitro* data showing that macrophages (2, 3, 5, 32, 33), smooth muscle cells (4), and alveolar macrophages (4) synthesize LPL, whereas endothelial cells do not (1, 6, 7). Although no *in situ* hybridization signal was seen over endothelial cells in any of the studied sections, immunostaining for LPL was positive over endothelial cells in many sections. Previous studies have shown that in normal guinea pig aortas LPL mRNA and protein were detected only in a small number of medial smooth muscle cells, whereas only immunoreactive LPL protein was found on endothelial cells (6). Studies in atherosclerotic human carotid arteries reported the presence of LPL immunoreactivity mainly in smooth muscle cells (13). Our findings demonstrate that lesion macrophages clearly express LPL mRNA and protein and could therefore contribute to the local production of LPL in early and late atherosclerotic lesions.

We have estimated that, at the sensitivity of the methods used, only 10–20% of the macrophages and smooth muscle cells in the lesions studied appear to express LPL mRNA, suggesting that only subpopulations of cells are responsible for the production of LPL. Lipid loading of cells, various cytokines, and growth factors have been reported to affect LPL expression in cultured (or isolated) macrophages (26–31). For example, tumor necrosis factor and interferon γ suppress and colony-stimulating factors stimulate the production of LPL by macrophages (26, 28–30). Thus, it is likely that local differences in the concentrations of these and other factors in atherosclerotic intima play an important role in the regulation of LPL gene expression.

What role could localized production of LPL play in the atherosclerotic process? (*i*) Because in early fatty streaks macrophages are located immediately adjacent to the abluminal endothelial surface, production of LPL by macrophages could lead to a localized high LPL concentration, which could then be quickly transposed to the luminal surface. This in turn could promote influx of remnants into the lesion area, as originally suggested by Zilversmit (8). (*ii*) If triglyceride-rich lipoproteins enter the intima, they could be degraded by LPL. Although large very low density lipoproteins (VLDLs) and chylomicrons may not be able to penetrate an intact endothelium, once the endothelium has been broken [as occurs over some fatty streaks (34, 35)] they would have easy access to the foam cells. Rapp *et al.* (36) have even isolated large VLDLs (35- to 80-nm diameter) from human plaques. Moreover, small VLDLs (or remnants) (35-nm diameter) have been demonstrated even in normal human aorta (12). (*iii*) Macrophages can take up even intact VLDLs, particularly VLDLs from certain hypertriglyceridemic patients (9). Although LPL secretion is not obligatory for this process, if LPL is produced in the lesions it could further degrade VLDL particles or remnants to still smaller particles, which, in turn, would be taken up still more rapidly by macrophages (37, 38). We have previously demonstrated that LPL secreted by macrophages in culture can almost completely hydrolyze the triglycerides of VLDLs (or chylomicrons) in the medium (37, 38). By utilizing apolipoprotein C-II-deficient VLDLs we demonstrated that uptake of the VLDL protein was in part LPL-dependent, since it was greatly accelerated by addition of apolipoprotein C-II. This led to cholesteryl ester and triglyceride enrichment of the

cells (38). In addition, we demonstrated that free fatty acids generated by this process could be taken up directly by macrophages and lead to enrichment not only in triglyceride but also in cholesteryl esters (38). We have also shown that macrophages possess a neutral triglyceride lipase activity, which probably leads to efficient hydrolysis and the ability of the macrophages to unload triglycerides (38, 39). Because macrophages mobilize triglycerides far more rapidly than cholesteryl esters (40), the continual uptake of remnants may lead eventually to net macrophage accumulation of cholesteryl esters, even though the remnants originally taken up were enriched in triglycerides. Finally, it should be noted that LPL modifies low density lipoprotein (LDL) so that it is more rapidly taken up by cultured macrophages (41). Thus, generation of LPL within the intima might accelerate the development of atherosclerotic lesions in several ways.

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