Lysosomal Enzymes Are Released From Cultured Human Macrophages, Hydrolyze LDL In Vitro, and Are Present Extracellularly in Human Atherosclerotic Lesions

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Objective—Human atherosclerotic lesions have been shown to contain lipid droplets and vesicles resembling those of in vitro enzymatically modified LDL. However, little is known about the hydrolytic enzymes in the arterial intima that induce fusion of LDL particles and so produce lipid droplets or that induce foam cell formation.

Methods and Results—Human coronary atherosclerotic lesions obtained at surgery and at autopsy were stained for lysosomal acid lipase and cathepsin D. The extracellular areas of macrophage-rich intimal regions of the atherosclerotic lesions stained positively for both cathepsin D and lysosomal acid lipase, whereas normal arteries were negative. When monocyte-derived macrophages were incubated with opsonized zymosan to stimulate the release of lysosomal enzymes from the cells and LDL was incubated with the macrophage-conditioned media, the apolipoprotein B-100, cholesteryl esters, and triacylglycerols of LDL were hydrolyzed. These hydrolytic modifications rendered the LDL particles unstable and induced their fusion. Cultured macrophages and smooth muscle cells took up the hydrolyase-modified LDL particles avidly and were transformed into foam cells.

Conclusions—Our in vivo and in vitro results suggest that lysosomal enzymes released from macrophages may induce hydrolytic modification of LDL and foam cell formation in the human arterial intima. (Arterioscler Thromb Vasc Biol. 2003;23:1430-1436.)

Key Words: macrophage ■ lysosomal enzymes ■ LDL ■ atherosclerosis

Atherogenesis is characterized by accumulation of LDL-derived lipids in the extracellular matrix of the arterial intima, recruitment of monocyte-derived macrophages, and their transformation into lipid-laden foam cells.1 The lipid accumulates extracellularly in atherosclerotic lesions in the form of small lipid droplets and vesicles.2 Modification of LDL in vitro by proteolysis, lipolysis, and oxidation has been shown to produce particles resembling those observed in the arterial intima.3-10 Lipid particles enriched in unesterified cholesterol have been isolated from the human arterial intima,11 and evidence for the presence of the particles resembling in vitro enzymatically modified LDL has been provided by the immunohistochemistry.12 However, the enzymes responsible for such LDL modification in the arterial intima have remained uncharacterized.

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Two enzymes that participate in the hydrolysis of endocytosed LDL in the lysosomal compartment of cells are cathepsin D and lysosomal acid lipase (LAL). The former initiates the lysosomal degradation of apolipoprotein B-100 (apoB-100)13 and is found in atherosclerotic intima.14,15 The latter is normally responsible for lysosomal hydrolysis of cholesteryl esters and triacylglycerols of the endocytosed LDL particles.16 Extracellular discharge of lysosomal enzymes is a common physiological response to a variety of inflammatory stimuli.17,18 Under pathologic conditions, cathepsin D has been found extracellularly in many tissues, eg, in the invading edge of the rheumatoid synovium,19 in the brain of patients suffering from Alzheimer disease,20 and in invasive human carcinomas.21 Moreover, Briozzo et al22 showed that cathepsin D is a major acidic protease secreted by breast cancer cells. LAL, in turn, is present in macrophage foam cells in the subendothelial area and deep in the intimal fibrous tissue,23 but there is little in vivo evidence of secretion of LAL. Recently, Buton et al24 suggested that LAL-mediated cholesteryl ester hydrolysis of extracellularly located and matrix-retained and aggregated LDL starts during the prolonged cell-surface contact, implying that enzymatically active LAL may have been released into the pericellular areas of activated macrophages.

In this study, we studied the presence and localization of cathepsin D and LAL in normal and atherosclerotic arterial...
intima and, moreover, studied modifications of LDL by lysosomal enzymes released by stimulated human macrophages.

**Methods**

An online Methods section is available at http://atvb.ahajournals.org.

**Results**

The presence of the lysosomal enzymes cathepsin D and LAL was studied by immunohistochemistry in normal and in atherosclerotic human coronary arteries obtained from autopsied and explanted hearts and compared with that of a lysosomal marker, the lysosome-associated membrane protein-1 (LAMP-1). Figure 1 shows immunostaining pattern that was typically found both in autopsy and transplant specimens. The amounts of cathepsin D and LAL in the cells of the normal arterial intima were so low that they were barely detectable by immunohistochemistry (panels A and C).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Immunohistochemistry of human coronary arteries. Cross-sections of grossly normal (left) and atherosclerotic (right) human coronary artery from explanted hearts were immunostained for cathepsin D (Cath D, A and B), LAL (C and D), macrophages (Mf, E and F), and LAMP-1 (G and H). Note the paucity of macrophages and the weak staining for lysosomal enzymes and LAMP-1 in the grossly normal sample and the strong immunostaining for lysosomal enzymes and LAMP-1 in the macrophage-rich area in the atherosclerotic coronary artery. I indicates intima; m, media; and a, adventitia.

However, as shown in panels B and D, in atherosclerotic lesions that contained large number of macrophages, the amounts of cathepsin D and LAL were dramatically increased, and both enzymes were colocalized with the macrophages (panel F). All of the sections stained with control antibodies (mouse-irrelevant isotype-specific Igs and rabbit IgG) were negative (not shown). Interestingly, in contrast to the mainly intracellular staining of LAMP-1 (panel H), cathepsin D and LAL were widely present extracellularly in the intima.

Next, we studied whether lysosomal hydrolases released from human monocyte-derived macrophages could modify LDL in vitro. As a model of stimulation-induced secretion of acid hydrolases, we incubated human macrophages with zymosan A opsonized with fresh human serum (Zop). Incubation of LDL with conditioned media derived from Zop-treated macrophages, but not from untreated control macrophages, led to generation of TCA-soluble radioactivity (Figure 2A) and free fatty acids (FFAs) (Figure 2B), indicating proteolysis and lipolysis of the LDL particles, respectively. This modification was not caused by oxidation, because the samples contained EDTA, the amount of thiobarbituric acid reactive substances (TBARS) in H-LDL

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Modification of LDL in macrophage-conditioned medium. 3H-LDL (20 µg) was incubated for the indicated times in 96-well plates with 175 µL of macrophage-conditioned media from Zop-stimulated (H-LDL) and unstimulated (control LDL) cells in 200 µL of buffer A. After the incubations, proteolysis of LDL was determined by measuring the TCA-soluble 3H radioactivity (A), the degree of lipolysis was determined by analyzing the amount of free fatty acids (B), and the turbidity of the reaction mixture was measured at 340 nm (C). Data shown are means of 3 independent experiments ± SEM. *P<0.05.
The proteolytic activity in the macrophage-conditioned medium was characterized with various protease inhibitors (Figure 3A). We found that of the various protease inhibitors studied, only pepstatin A, an inhibitor of aspartic proteinases, fully abolished the proteolytic activity of the macrophage-conditioned medium. We also found that immunodepletion of the macrophage-conditioned media with antibody against cathepsin D blocked LDL proteolysis (Figure 3B), demonstrating that hydrolysis of apoB-100 depends on cathepsin D activity. Finally, the patterns of apoB-100 fragmentation of LDL were compared in SDS-PAGE. As shown in Figures 3C and 3D, only commercial cathepsin D produced the degradation pattern of LDL similar to the pattern induced by the macrophage-conditioned media. Taken together, the data suggest a major role for cathepsin D in the proteolytic modifications of LDL.

Analysis of the lipid composition of the H-LDL particles revealed that the amounts of cholesteryl esters (CEs) and triacylglycerols (TGs) were significantly decreased and the amounts of unesterified cholesterol (UC) and FFAs were significantly increased in H-LDL (Figure 4A). No significant hydrolysis of phosphatidylcholine or sphingomyelin was found. To confirm that LDL lipids were hydrolyzed by LAL, LAL was removed from the macrophage-conditioned medium by immunoprecipitation. It was found that immunodepletion of LAL from the macrophage-conditioned media totally inhibited release of free fatty acids from the LDL lipids (Figure 4B).

To study the forms of cathepsin D and LAL that are secreted on treatment of human macrophages with Zop, macrophage-conditioned media were studied by Western blot analysis. As shown in Figure 5, the conditioned medium from unstimulated macrophages contained a single immunopositive 32-kDa band corresponding to the pro-form of ca-
thepsin D. In addition to the 52-kDa band, conditioned medium from Zop-treated macrophages contained 48-kDa and 32-kDa bands corresponding to the catalytically active intermediate proenzyme and the mature forms of cathepsin D, respectively.28 Conditioned medium from Zop-treated macrophages also contained large amounts of LAL. In addition to the 49-kDa band, LAL also appeared as the 41-kDa band, representing glycosylated and deglycosylated forms of the enzyme, respectively.29

Next we studied uptake of H-LDL by macrophages in comparison with native LDL, acetylated LDL (acLDL), and oxidized LDL (oxLDL). Figures 6A through 6D show mouse peritoneal macrophages incubated for 18 hours with LDL, acLDL, oxLDL, or H-LDL and then stained with Oil Red O. No lipid staining was found in the LDL-treated cells (panel A), whereas typical intracellular lipid droplets were found in the macrophages incubated with acLDL (panel B), oxLDL (panel C), and H-LDL (panel D). Analysis of the cholesterol content of mouse macrophages (Figure 6E) showed that the intracellular contents of both UC and CE were significantly increased in H-LDL–treated cells (H-LDL) compared with resting cells (B), whereas incubation of the cells with acLDL significantly increased the intracellular content of CE but not UC. Importantly, incubation of human macrophages with H-LDL and acLDL (Figure 6F) also significantly increased intracellular contents of both UC and CE. Macrophage-conditioned medium, in the absence or presence of native LDL, did not increase the cholesterol content of the macrophages (not shown). Electron microscopy (Figure II, please see the online supplement at http://atvb.ahajournals.org) revealed that H-LDL–treated human macrophages had large amounts of modified lipoproteins in compartments resembling phagosomes and also contained cytoplasmic lipid inclusions. The mechanisms of uptake of H-LDL by macrophages was studied by competition experiments. In the presence of 20-fold excess of unlabeled lipoproteins, the degradation of H-LDL by macrophages was inhibited by 53±12% with LDL, by 59±12% with acLDL, by 69±1% with mildly (3-hour) oxidized LDL, by 57±35% with totally (18-hour) oxidized LDL, and by 67±12% with the unlabeled H-LDL. This suggests that the uptake of H-LDL occurs through both scavenger receptors and the LDL receptor.

Finally, cultured human coronary artery smooth muscle cells (HCASMCs) were incubated for 72 hours in the absence or presence of LDL, acLDL, oxLDL, or H-LDL and stained with Oil Red O (Figure 7). Numerous lipid droplets were seen in the cytoplasm of H-LDL–treated HCASMCs (panel D) but not in that of cells treated with LDL, acLDL, or oxLDL (panels A, B, and C). The results are consistent with previous studies showing little expression of scavenger receptors in resting smooth muscle cells in culture and in normal arteries30,31 and suggest that in smooth muscle cells the uptake of H-LDL is mediated by the LDL receptor.

**Discussion**

The present study shows that 2 lysosomal enzymes, cathepsin D and lysosomal acid lipase, are present in human atherosclerotic lesions. The lysosomal enzymes showed both punctate cellular and diffuse extracellular staining, whereas LAMP-1 showed mainly punctate cellular staining. This observation supports the notion that the enzymes were present not only in the lysosomes but also extracellularly in the human atherosclerotic lesions. To exclude that this finding results from postmortem release of these enzymes, not only autopsy material but also samples obtained at surgery were examined. The paucity of extracellular staining of LAMP-1 reveals that the lysosomes are intracellular and have remained intact, suggesting that rather than being passively released from dying cells, the lysosomal enzymes were secreted actively by living cells. On the basis of their colocalization with macrophage infiltrates, it is also evident that the lyso-
Somat enzymes were synthesized and secreted chiefly by the macrophages present in the lesions. Consistent with our results, other lysosomal enzymes have also been found extracellularly, e.g., sphingomyelinase in human atherosclerotic lesions and cathepsins B and D in amyloid deposits in Alzheimer brains. Moreover, cathepsins K, B, S, L, and D have been shown to be secreted simultaneously by cultured monocyte-derived macrophages. Taken together, the present and previous findings suggest that when the macrophages appear and the atherosclerotic lesions start to develop, several different lysosomal enzymes are present extracellularly in the human arterial intima.

To be capable of modifying the LDL particles, the released enzymes must be catalytically active. Most mammalian cells release precursor forms of the enzymes that are ultimately targeted to lysosomes. The precursors are taken up by the secreting cells themselves or by the neighboring cells via mannose-6-phosphate receptors located on the cell surface and are then targeted to the lysosomes for final maturation. Importantly, specialized phagocytes, notably neutrophils and macrophages, also release mature, i.e., catalytically active, lysosomal enzymes, a property related to their competence in host defense and in the generation of local inflammation. In our present in vitro system using human monocyte-derived macrophages, both Western blot analysis and functional studies with LDL indicated that cathepsin D and lysosomal acid lipase were secreted in catalytically active form.

Various agents that have been shown to trigger the release of lysosomal enzymes from macrophages, i.e., asbestos, streptococcal cell wall substance, zymosan, and immuno-

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**Figure 6.** Uptake of modified LDL by mouse peritoneal macrophages and analysis of the cholesterol content of H-LDL–treated macrophages. Mouse macrophages were plated on glass coverslips and incubated for 18 hours in the presence of 40 μg/mL of native LDL (LDL) (A), acLDL (B), LDL oxidized with 5 μmol/L CuSO₄ for 18 hours (oxLDL) (C), or LDL that was modified with conditioned medium from Zop-treated macrophages (H-LDL) (D). After incubation, the cells were stained with Oil Red O and counterstained with hematoxylin. The micrographs shown are representative of 4 independent experiments. Mouse peritoneal macrophages (E) and human monocyte-derived macrophages (F) were treated as described above, and after incubation, the cellular lipids were extracted and analyzed by HPTLC. The amounts of unesterified cholesterol and cholesteryl esters were normalized to the total protein content of the cells. Data shown are means of 4 to 7 independent experiments ± SEM. *P<0.05 vs control cells (Bl), **P<0.01 vs Bl.

**Figure 7.** Uptake of H-LDL by HCASMCs. HCASMCs were cultured on coverslips, and their growth was arrested for 48 hours. The cells were then incubated for 72 hours with 20 μg/mL of native LDL (LDL), acetylated LDL (acLDL), LDL oxidized with 5 μmol/L CuSO₄ for 18 hours (oxLDL), or (H-LDL). After incubation, the cells on the coverslips were stained with Oil Red O and counterstained with hematoxylin. The micrographs shown are representative of 2 independent experiments.
complexes, all cause macrophages to also exert phagocytic activity. In the present study, we used serum-opsonized zymosan particles to induce secretion of enzymes by the macrophages. Serum opsonization leads to coating of the particles with complement C3b and immunoglobulins. By binding to their respective receptors (the complement receptor CR3 [CD11b/CD18] and the Fcγ receptors on the macrophages), these ligands trigger phagocytosis with ensuing efficient secretion of lysosomal enzymes. Importantly, immunoglobulins, in the form of LDL-immunocomplexes, and, as a result of local complement activation, C3b is deposited in the human atherosclerotic intima and are among the potential triggers of release of lysosomal enzymes in the lesions.

The pH in the normal arterial intima is near neutral, whereas the pH in lysosomes and the optimum pH of lysosomal enzymes is acidic. Accordingly, a major question is whether the pH locally in atherosclerotic lesions allows catalytic activity of the lysosomal enzymes. Macrophages are able to acidify their pericellular environment with the aid of proton pumps and by secreting lactic acid into the extracellular space. Atherosclerotic lesions are characterized by chronic inflammation, and the pH of the extracellular fluid in inflammatory sites is known to be acidic. Indeed, as signs of an acidic environment, atherosclerotic lesions have been shown to contain accumulations of lactic acid and hypoxia has been demonstrated by the low oxygen tension, the positive staining with the hypoxia marker theophylline, and the presence of neovascularization. Thus, there is substantial evidence that the pH of the extracellular milieu may be acidic in atherosclerotic lesions, at least in inflammatory infiltrates, potentially allowing extracellular action of lysosomal enzymes.

Taken together, the present study reveals the possibility that the lysosomal enzymes cathepsin D and LAL released from macrophages participate in the modification of LDL in atherosclerotic lesions. These observations suggest novel candidate enzymes for generating hydrolyase-modified LDL in atherosclerotic lesions. Such hydrolytically modified LDL particles may generate extracellular lipid droplets and vesicles and cause both extracellular and intracellular accumulation of lipid in the arterial intima.

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References

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