Enzymatic Modification of Low-Density Lipoprotein in the Arterial Wall

A New Role for Plasmin and Matrix Metalloproteinases in Atherogenesis

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Objective—Functionally interactive proteases of the plasminogen/plasmin and the matrix metalloproteinase (MMP) system degrade and reorganize the extracellular matrix of the vessel wall in atherosclerosis. Here we investigated whether such proteases are able to confer atherogenic properties onto low density lipoprotein by nonoxidative modification.

Methods and Results—Similar to the recently described enzymatically-modified low-density lipoprotein (E-LDL), native LDL exposed to plasmin or matrix MMP-2 or MMP-9 and cholesterylester-hydrolase (CEH) showed extensive deesterification, with ratios of free cholesterol to total cholesterol rising to 0.8 compared with 0.2 in native LDL. When the ratio exceeded 0.6, both plasmin/CEH-LDL and MMP/CEH-LDL fused into larger particles. In parallel, they gained C-reactive protein–dependent complement-activating capacity. E-LDL produced with any protease/CEH combination was efficiently taken up by human macrophages, whereby marked induction of MMP-2 expression by E-LDL was observed. These in vitro findings had their in vivo correlates: urokinase-type plasminogen activator, MMP-2, and MMP-9 were detectable in both early and advanced human atherosclerotic lesions in colocalization with E-LDL.

Conclusions—Plasmin and MMP-2/MMP-9 may not only be involved in remodeling of the extracellular matrix in progressing plaques, but they may also be involved in lipoprotein modification during genesis and progression of atherosclerotic lesions. (Arterioscler Thromb Vasc Biol. 2004;24:1-7.)

Key Words: atherosclerosis ■ lipoproteins ■ macrophages ■ metalloproteinases ■ plasminogen activators

It is widely held that atherogenesis is triggered by enhanced entrapment of low-density lipoprotein (LDL) in the intima, which is followed by its uptake by macrophages. Both oxidative and nonoxidative processes can generate potentially atherogenic LDL derivatives.1 We are pursuing the concept that enzymatic remodeling of the lipoprotein is a key modification, because proteolytic cleavage of apolipoprotein B (apoB) in conjunction with hydrolysis of cholesteryl esters generates lipoprotein particles that are similar to lesion-derived LDL in structure, biological properties, and composition.2–3 Enzymatically-remodeled LDL (E-LDL) binds C-reactive protein (CRP) and activates complement.4 E-LDL induces foam cell formation in monocytes,5 macrophages,3 and smooth muscle cells,6 stimulates MCP-1 production,7 and directly promotes adhesion and transmigration of monocytes through endothelial cell monolayers.8 These in vitro findings have their in vivo correlates: immunohistological analyses with specific monoclonal antibodies (mAbs) have revealed extensive extracellular deposits of E-LDL at the early stages of atherosclerotic lesion formation.9 CRP and activated complement components are also present in colocalization with E-LDL.4,9 Like in vitro–generated E-LDL, lesioned LDL has a high content of free cholesterol;10–12 therefore, it is apparent that extensive deesterification of cholesteryl esters must indeed occur in the lesions.

We used trypsin in combination with cholesteryl esterase to produce E-LDL in vitro.3 Although both neutral and acid cholesteryl ester hydrolase have been demonstrated to be present in the arterial vessel wall of humans and rabbits13,14 and the concentration of the hydrolase is so high in atherosclerotic lesions that it can be directly detected by immunohistochemistry,15 it remains unclear which protease might be acting in synergy with this enzyme to generate E-LDL in vivo. Most recently, we found that macrophages are stimulated by E-LDL to produce cathepsin H, that cathepsin H can replace trypsin as the modifying protease, and that cathepsin H colocalizes with E-LDL in atherosclerotic lesions.16 Thus, the idea is emerging that the specificity of a modifying protease bears little relevance for the creation of atherogenic E-LDL. Subsequent generation of free cholesterol and free...
fatty acids leads to fusion of E-LDL, which is endowed with the characteristic atherogenic properties. Should this hypothesis be correct, several proteases might be involved in generating E-LDL in situ. Establishing the redundancy of a basic process in atherogenesis would obviously be of interest.

Here, we report the analysis of 3 enzymes known to be present in atherosclerotic lesions, namely plasmin, metalloproteinase-2 (MMP-2), and MMP-9, for their potential to modify LDL nonoxidatively and confer potential proatherogenic properties to the lipoprotein particle.

Methods
The Methods section can be found in an online supplement available at http://atvb.ahajournals.org.

Isolation and Modification of LDL
LDL from healthy subjects, aged 18 to 65 years, was isolated by preparative ultracentrifugation (d=1.020 to 1.062 g/mL). Concentrations refer to the total cholesterol concentration in the lipoprotein samples.

For enzymatic modification, LDL (5 to 7 mg/mL) was incubated in veronal buffered saline (VBS) with a protease at 37°C for 18 hours, followed by further incubation with cholesteryl ester hydrolase (CEH; Sigma). Reactions were monitored by measuring the absorbance of supernatants at 412 nm. Curves obtained from the hemolysis readings were inverted to depict complement consumption.

C3-Conversion
C3-conversion in human serum was assessed by 2D quantitative immunoelectrophoresis as described. Briefly, lipoproteins were added to human serum (final concentration: 400 μg/mL cholesterol; ≤10 μg/mL recombinant CRP). After 4 hours at 37°C, samples were subjected to 2D immunoelectrophoresis. C3-turnover was assessed by planimetry of the areas delimited by the C3 and C3b/C3c arcs.

Monocyte Isolation and Cell Culture
Monocytes were isolated from buffy coats of healthy donors as described and cultured for 7 days in medium supplemented with 10% AB serum. Cells were then incubated for 16 hours in the presence of lipoproteins at a concentration of 100 μg cholesterol/mL, washed twice, and subsequently fixed in 10% formaldehyde. For enzymatic modification of LDL (data not shown), monocytes were incubated with cholesteryl ester hydrolase (CEH; Sigma) and MMP-9 (1 μg/mL; Sigma), and MMP-9 (1 μg/mL; Sigma).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and Western Blot Analyses
Monocytes from 4 healthy donors were isolated and incubated with native or modified LDL for 24 hours. Thereafter, cells were harvested and lysed in sodium dodecyl sulfate (SDS)-loading buffer. Proteins (from 10⁶ cells; Figure 3B) or lipoprotein samples, respectively, were separated in 10% SDS polyacrylamide gels, transferred to nitrocellulose membranes, and probed with primary antibodies for MMP-2, MMP-9, or E-LDL. Blots were developed by enhanced chemiluminescence.

Results
LDL Modified With Plasmin, MMP-2 or MMP-9, and CEH is Similar to LDL Modified with Trypsin
Enzymatic transformation of LDL to potentially atherogenic E-LDL has previously been achieved by combined treatment with trypsin and CEH. It was then of interest to discern whether plasmin or MMP could replace trypsin. Both plasmin/CEH-LDL and MMP/CEH-LDL displayed marked electronegativity in agarose gel electrophoresis, similar to trypsin-generated E-LDL (not shown). E-LDL preparations were analyzed for their content of free and esterified cholesterol. In all cases, the molar ratio of free to total cholesterol rose from ~0.2 to reach ~0.8, as previously found for both trypsin- and cathepsin H-modified LDL and for lesion-derived LDL. Deesterification was not noted when 1 of the 2 enzymes was omitted. Furthermore, we performed 1 experiment with tissue inhibitor of metalloproteinase-1 (TIMP-1) and found that TIMP-1 inhibited MMP-9-mediated enzymatic modification of LDL (data not shown). Taken together, these results confirmed the report that proteolytic nicking of apoB renders cholesterol esters accessible to the action of CEH.

LDL Modified With Plasmin, MMP-2 or MMP-9, and CEH has CRP-Dependent Complement-Activating Capacity
The ability of different LDL-preparations to activate complement was analyzed by 2D immunoelectrophoresis of C3. Figure 1 depicts results obtained with 50 μg/mL native LDL, plasmin/CEH-LDL, MMP-2/CEH-LDL, or MMP-9/CEH-LDL. Low basal C3 turnover of 10% to 20% occurred in samples spiked with native LDL in the absence or presence of CRP. Plasmin/CEH-LDL, MMP-2/CEH-LDL, or MMP-9/CEH-LDL alone slightly augmented C3 cleavage. However, when CRP was added to the modified LDL preparations, C3 consumption was markedly enhanced to ~50% to 70%. No enhancement of complement activation was observed with control serum samples incubated in buffer alone, in serum spiked with the enzyme mix, in CRP without lipoproteins, and when either protease or CEH was omitted. Dose-response and kinetic experiments were undertaken using the combination of plasmin and CEH or plasmin alone.
First, the protease concentration was varied from 0 to 2 U/mL. After overnight incubation, CEH was applied at 20/HL9262 g/mL for another 10 to 48 hours. Formation of E-LDL was monitored by assessment of cholesterol deesterification, turbidity measurements, and complement consumption tests. It was found that as little as 0.0008 U/mL plasmin sufficed to promote formation of functional E-LDL. At this lowest concentration, SDS-PAGE revealed that very limited nicking of apoB had occurred, leading to appearance of several high molecular weight products (>200 kDa) and one major product of lower molecular weight (~75 kDa). Plasmin alone was used at 0.2 U/mL. The respective SDS-PAGE did not differ from the one using plasmin/CEH-LDL at 0.2 U/mL. (Figure I, available online at http://atvb.ahajournals.org).

Kinetic experiments were then performed using 0.02 U/mL plasmin. In these experiments, LDL preparations were reequilibrated in VBS before enzyme treatment so that complement activation tests could be performed on aliquots taken at different times. Deesterification was followed after addition of CEH, and it was found that the ratio of free to total cholesterol rapidly rose from 0.2 to reach 0.5 to 0.6 within 15 minutes. Thereafter, deesterification proceeded slowly and the lipoprotein solutions remained clear until the ratio of free to total cholesterol exceeded 0.6. At varying times thereafter, solutions became turbid because of lipoprotein fusion. In the absence of CRP, complement activation occurred at high E-LDL concentrations. In the presence of CRP, complement consumption was noted already at low E-LDL concentrations. In A through C, results are representative of 3 separate experiments.
Functional tests revealed that complement-activating function correlated with the fusion step. Thus, when a sample from the foregoing experiment was tested at 1 hour, during which extensive deesterification had already occurred, no complement consumption was discerned with or without CRP (Figure 2B). In contrast, once an LDL preparation had become turbid, spontaneous complement consumption (in the absence of added CRP) was observed at high E-LDL concentrations of >100 µg/mL. However, complement consumption could be triggered at lower E-LDL concentrations by addition of 5 µg/mL CRP (Figure 2C). This activation pattern was found for all E-LDL preparations (plasmin/CEH-LDL, MMP-2/CEH-LDL, and MMP-9/CEH-LDL) without exception.

Enhanced Macrophage Uptake of LDL Modified With Plasmin, MMP-2 or MMP-9, and CEH
Plasmin/CEH-LDL, MMP-2/CEH-LDL, and MMP-9/CEH-LDL induced foam cell formation as revealed by oil red staining (not shown). Results of quantification of cellular cholesterol are shown in Figure 3A. Incubation of cells with 100 µg/mL (cholesterol) native LDL for 16 hours caused a slight but statistically not significant increase of total cellular cholesterol (~10%). In contrast, incubation with either plasmin/CEH-LDL, MMP-2/CEH-LDL, or MMP-9/CEH-LDL led to massive cholesterol accumulation. Total cellular cholesterol content increased 2- to 4-fold, corresponding to a >10-fold higher cholesterol uptake compared with that found with native LDL. Foam cell formation was not noted if 1 of the enzymes was omitted.

MMP-2 Protein Expression in Monocytes Is Increased by E-LDL
To investigate whether MMP-2/CEH-LDL or MMP-9/CEH-LDL induce MMP-2 or MMP-9, monocytes were treated with lipoprotein preparations and analyzed by Western blot using specific antibodies directed against MMP-2 and MMP-9. Expression of MMP-2 was enhanced in MMP-2/CEH-LDL-treated cells compared with LDL-treated cells, a result that was confirmed in 4 donors. Figure 3B (top) depicts a representative blot; control incubations without lipoprotein were additionally used for comparison. In contrast, expression of MMP-9 (bottom) was not different in MMP-9/CEH-LDL-treated cells compared with LDL-treated cells.

Plasmin, MMP-2, and MMP-9 Colocalize With E-LDL in Human Atherosclerotic Lesions
We then sought evidence for the presence of MMP-2 and MMP-9 using a specific mouse mAb against these enzymes and the plasmin cascade using a specific mouse mAb against the uPA in human coronary atherosclerotic lesions. Tissue sections were stained for uPA, MMP-2, and MMP-9 and compared with E-LDL staining. The predominant manifestation of E-LDL deposits in an early lesion (Figure 4D) was a diffuse deposition in the deep fibroelastic and fibromuscular layers of the intima adjacent to the media as described previously.4,9 There was a close association and overlapping of E-LDL and uPA (Figure 4A) with MMP-2 (Figure 4B) and MMP-9 (Figure 4C) epitopes within the deeper portion of the intima. In a more advanced atherosclerotic lesion (Figure 4F through 4J), uPA (Figure 4F), MMP-2 (Figure 4G), and MMP-9 (Figure 4H) also colocalized with the E-LDL epitopes (Figure 4I). The proteases and E-LDL were diffusely scattered among the extracellular components of the lipid cores of lesions, predominantly in the central part. In addition, intracellular staining for uPA, MMP-2, and MMP-9 of foam cells was evident. As reported previously, these cells did not stain positively for E-LDL, which was likely because of destruction of the neoepitope in the course of extensive proteolysis, as has been demonstrated in vitro.9 Control staining performed with the irrelevant isotype-matched antibodies yielded negative results with all tissue specimens (Figure 4E and 4J).
MAb AIL-3 Recognizes LDL After Modification With Plasmin, MMP-2 or MMP-9, and CEH

The anti–E-LDL antibody AIL-3, which was derived against LDL modified by trypsin and CEH,9 also reacted with E-LDL generated by incubation with plasmin, MMP-2, or MMP-9, respectively. Equal amounts of primary antibody (1:5000 dilution) were added per well. The amount of antibody bound was then measured with alkaline phosphatase–labeled goat anti-mouse IgG using chemiluminescent technique. Luminescence was measured as number of flashes of light (RLU indicates relative light units). Means and error bars (SD) are calculated from 3 different experiments.

The chemiluminescence immunoassay, a band was not seen with MMP-2/CEH-LDL and MMP-9/CEH-LDL. This probably reflects a loss of the epitope after SDS-polyacrylamide gel electrophoresis.

Discussion

Ample evidence indicates MMPs can weaken the fibrous cap of arterial plaques and thereby make them prone to rupture.22,23 The plasminogen cascade represents another proteolytic system involved in the genesis and progression of atherosclerotic lesions. Its contribution to the development of experimental neointimal lesions after injury and to aortic medial destruction was demonstrated in uPA and plasminogen activator inhibitor-1–null mice24 and apoE–null mice,25 respectively. Despite this detrimental role, the overall effect of MMPs and the plasminogen cascade in the pathogenesis of atherosclerosis and its sequelae is not entirely clear. New insights obtained from recent studies with MMP inhibitors and genetic manipulation also point to beneficial effects of MMPs in the process.23,26

Here we report another role for MMPs and plasmin in the arterial vessel wall. Both proteases can degrade LDL and trigger its conversion to a CRP-binding, complement-activating particle. E-LDL with atherogenic properties was originally produced in vitro by the consecutive action of trypsin, CEH, and neuraminidase.3 The latter enzyme was needed to confer maximal complement activating properties to E-LDL. With the discovery that LDL modified by trypsin and CEH could bind CRP, converting it to a very potent complement activator,4 incubation with neuraminidase was omitted. Trypsin-generated E-LDL is similar to lesion derived lipoprotein remnants in having a high content of unesterified cholesterol and a tendency to fuse and form heterogeneously-sized lipidic droplets. Furthermore, in vitro–generated E-LDL has neoepitopes that are detectable in lesion.
areas rich in extracellular lipids. Like E-LDL, lesion-derived material also activates complement, concurring with the fact that complement activation is one of the first steps in lesion initiation, even preceding monocyte infiltration.27

The now 8-year-old concept that we have been pursuing proposes extracellular enzymatic degradation of LDL to represent a key step leading to generation of an atherogenic lipoprotein.3,28,29 One of the most prevalent criticisms of this concept has been the fact that trypsin is an intestinal enzyme with no connection to atherosclerotic lesion formation. After the demonstration that cathepsin H can substitute for trypsin in generating E-LDL, the present observation that MMPs and plasmin are similarly effective may resolve an issue of basic importance. LDL treated with any protease followed by incubation with CEH shows the same properties as trypsin/CEH-treated LDL. E-LDL is highly enriched in unesterified cholesterol, binds CRP to activate complement, and efficiently drives foam cell formation. Finally, as demonstrated by chemiluminescence immunoassay or Western blotting, it shares the epitope for the E-LDL specific mAb AIL-3, which has previously been used to stain nonoxidized modified LDL by chemiluminescence immunoassay or Western blotting, it shares the epitope for the E-LDL specific mAb AIL-3, which has previously been used to stain nonoxidized modified LDL with no connection to atherosclerotic lesion formation. After the demonstration that cathepsin H can substitute for trypsin in generating E-LDL, the present observation that MMPs and plasmin are similarly effective may resolve an issue of basic importance. LDL treated with any protease followed by incubation with CEH shows the same properties as trypsin/CEH-treated LDL. E-LDL is highly enriched in unesterified cholesterol, binds CRP to activate complement, and efficiently drives foam cell formation. Finally, as demonstrated by chemiluminescence immunoassay or Western blotting, it shares the epitope for the E-LDL specific mAb AIL-3, which has previously been used to stain nonoxidized modified LDL in early human atherosclerotic lesions.9 Thus, E-LDL generated by all tested proteases are endowed with the same atherogenic properties as the originally described trypsin-generated E-LDL.

The present study provides new information on the in vitro formation of functional E-LDL, and a number of points merit consideration. It has become clear that very limited nicking of the apoB "cage," unrelated to any specific apoB breakdown patterns, suffices to render the underlying cholesteryl esters accessible to CEH. This accords with the finding that any one of the tested proteases can assume the modifying function; that is, that specificity plays no recognizable role. Second, the kinetic experiments reveal that CRP binding and complement activating function correlate not with the onset of deesterification but with the fusion step that occurs after extensive scission of the cholesteryl esters has occurred. This finding is in line with the fact that neither native nor oxidized LDL possess the complement-activating properties. We have proposed that complement activators are present but masked in native LDL molecules, and that they become exposed when the lipoprotein is entrapped in tissues.29,30 This is thought to primarily subsist a physiological function, because innate immune mechanisms can thus be recruited to remove the lipoprotein with its insoluble cargo. Exposure of the activators requires fusion of E-LDL particles, which occurs only by the combined action of proteases (like plasmin, MMP-2, or MMP-9) and CEH. Thus, the action of plasmin and MMPs is not thought to be primarily atherogenic. Fusion of E-LDL particles is not equivalent to lipoprotein aggregation, which can occur after massive oxidation. The time required for fusion to occur was found to vary considerably among the LDL preparations. The reasons for this are presently unknown, but one possibility is that differences in LDL subfractions play a role.31,32

Evidence for the presence of active MMPs and plasmin in atherosclerotic lesions is abundant,33–35 and MMPs and plasmin interact functionally and cooperate in extracellular matrix degradation.23,24,36 Plasmin plays a role in media destruc-


