Plasmin Promotes Foam Cell Formation by Increasing Macrophage Catabolism of Aggregated Low-Density Lipoprotein

Abigail S. Haka,* Inna Grosheva,* Rajesh K. Singh, Frederick R. Maxfield

Objective—The plasmin/plasminogen system is involved in atherosclerosis. However, the mechanisms by which it stimulates disease are not fully defined. A key event in atherogenesis is the deposition of low-density lipoprotein (LDL) on arterial walls where it is modified, aggregated, and retained. Macrophages are recruited to clear the lipoproteins, and they become foam cells. The goal of this study was to assess the role of plasmin in macrophage uptake of aggregated LDL and foam cell formation.

Approach and Results—Plasminogen treatment of macrophages catabolizing aggregated LDL significantly accelerated foam cell formation. Macrophage interaction with aggregated LDL increased the surface expression of urokinase-type plasminogen activator receptor and plasminogen activator activity, resulting in increased ability to generate plasmin at the cell surface. The high local level of plasmin cleaves cell-associated aggregated LDL, allowing a portion of the aggregate to become sequestered in a nearly sealed, yet extracellular, acidic compartment. The low pH in the plasmin-induced compartment allows lysosomal enzymes, delivered via lysosome exocytosis, greater activity, resulting in more efficient cholesteryl ester hydrolysis and delivery of a large cholesterol load to the macrophage, thereby promoting foam cell formation.

Conclusions—These findings highlight a critical role for plasmin in the catabolism of aggregated LDL by macrophages and provide a new context for considering the atherogenic role of plasmin. (Arterioscler Thromb Vasc Biol. 2013;33:1768-1778.)

Key Words: aggregated low-density lipoprotein ■ atherosclerosis ■ fibrinolysin ■ foam cells ■ low-density lipoprotein cholesterol ■ macrophages

Multiple lines of evidence suggest that the plasmin/plasminogen system is involved in the initiation and progression of atherosclerosis.1–9 Plasmin is a fibrinolytic serine protease capable of directly degrading components of the extracellular matrix and activating matrix metalloproteinases.10–12 Local increases in fibrinolytic activity associated with human atherosclerotic vessels were originally reported >40 years ago13 and have since been confirmed.1–4,10 Plasmin is generated via the cleavage of plasminogen by either urokinase-type plasminogen activator (uPA) or tissue PA. Monocytes/macrophages are a major source of uPA in atherosclerotic lesions10 where it is synthesized in an immature form, known as pro-uPA.14 Pro-uPA becomes activated when it binds to the cell surface receptor uPA receptor (uPAR), thereby accelerating the conversion of plasminogen to plasmin.15 In spite of extensive research, the role of the plasmin/plasminogen system in atherosclerotic lesion formation and progression remains incompletely characterized.

Studies in both humans and mouse models indicate an atherogenic role for plasmin.1 In several large clinical studies, elevated plasma concentrations of both plasmin and its precursor plasminogen were shown to be risk factors for both atherosclerosis and myocardial infarcts.2–4 Further, the expression of uPA10,16,17 and uPAR18,19 is upregulated in atherosclerotic lesions and correlates with disease severity.16,18 Immunolocalization analyses have demonstrated uPAR staining localized to macrophages in the neointima of atherosclerotic lesions, but little uPAR is found on macrophages in nonatherosclerotic arteries.19 Further, macrophage-targeted uPA overexpression is atherogenic in both ApoE−/− and LDLR−/− mice,6,8 whereas macrophage-specific uPA knockout is atheroprotective in ApoE−/− mice.9 These data strongly support the hypothesis that elevated uPA expression by artery wall macrophages accelerates atherosclerosis. Despite the fact that both animal and human studies have indicated a key role for macrophages in the atherogenic mechanisms of the plasmin/plasminogen system, most research has focused on plasmin-mediated modification of the extracellular matrix,20,21 As such, a coherent picture of the role of the plasmin/plasminogen system in atherogenesis, in particular with respect to macrophage biology, has not yet emerged.
Our laboratory is interested in the conversion of macrophages to foam cells via their interaction with aggregated low-density lipoprotein (agLDL). Although most studies examine foam cell formation via the incubation of macrophages with modified monomeric LDL (eg, oxidized LDL), this does not accurately reflect the in vivo environment because the vast majority of the LDL in atherosclerotic plaques is aggregated and avidly bound to the subendothelial matrix. For example, >90% of lesional lipoproteins in human aortic fatty streaks were not released by extraction or by electrophoresis, and monocyte/macrophage interaction with agLDL in atherosclerotic lesions has been visualized with electron microscopy. Thus, mechanisms of foam cell formation based on ingestion of aggregated, rather than monomeric LDL, may be more physiologically relevant.

Previous studies in our laboratory and others have elucidated a novel pathway for macrophage foam cell formation via catabolism of agLDL. We have shown that when macrophages come into contact with LDL aggregates, an extracellular, acidic, hydrolytic compartment (a lysosomal synapse) is formed. Lysosomes are delivered to the lysosomal synapse via targeted exocytosis, which results in the hydrolysis of LDL cholesteryl esters (CEs) and transfer of free cholesterol (FC) to the macrophage with subsequent foam cell formation. The Kruth laboratory has examined the effects of plasmin on macrophages interacting with agLDL. They found that plasmin treatment can disaggregate and release much, but not all, of the agLDL contained in the lysosomal synapse (also called a surface-connected compartment), generating lipoprotein structures similar to those observed extracellularly in atherosclerotic lesions. It is likely that the plasmin-mediated release of agLDL is caused by degradation of apolipoprotein B.

In this study, we examine the effects of plasmin on the interaction between macrophages and aggregated lipoproteins. Rather than examining the aggregate released by plasmin treatment, we focus on the portion of the aggregate that is not released from the lysosomal synapse and remains cell associated. Surprisingly, we found that plasmin treatment of macrophages interacting with agLDL caused a significant increase in foam cell formation. Incubation of macrophages with agLDL increased the surface expression of uPAR and PA activity, which would produce a high level of plasmin near the cell surface. To understand the mechanism by which plasmin promotes foam cell formation, we visualized the effects of plasmin treatment on macrophage agLDL interactions using several microscopy and biochemical techniques. These experiments indicate that plasmin cleaves cell-associated agLDL, resulting in changes in the morphology of the lysosomal synapse and allowing a portion of the aggregate to be sequestered in a nearly sealed, yet extracellular, actin-dependent, acidic compartment. The morphological changes in the compartment induced by plasmin facilitate generation of a more acidic environment, which in turn allows lysosomal enzymes greater activity. This results in more efficient CE hydrolysis and the delivery of a large cholesterol load to the macrophage. These findings indicate that physiological plasminogen concentrations are sufficient for plasmin-mediated agLDL processing and provide a mechanism for the ability of plasmin to accelerate foam cell formation and atherosclerosis. A detailed understanding of the mechanisms of foam cell formation is imperative for successful therapeutic targeting of atherosclerosis.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**Macrophage Incubation With Plasminogen Accelerates Foam Cell Formation**

To examine the effects of plasmin on macrophage uptake of agLDL, bone marrow–derived macrophages interacting with AlexaFluor-546 (Alexa546)-agLDL were incubated for 4 hours in the absence (Figure 1A) or presence of a physiological concentration of plasminogen without (Figure 1B) or with (Figure 1C) α2-antiplasmin treatment. After agLDL incubation, cells were fixed and labeled with LipidTOX Green to detect lipid droplets. LipidTOX Green will also stain the CE portion of the agLDL, so aggregates appear yellow/orange. In the absence of plasminogen, most agLDL remained extracellular after 4 hours of incubation, and only a small percentage of cells containing LipidTOX-positive droplets could be seen (Figure 1A). However, bone marrow–derived macrophages incubated with agLDL in the presence of plasminogen resulted in the formation of numerous cells containing neutral-lipid droplets (Figure 1B, arrows), indicative of foam cell formation. The percentage of LipidTOX-positive cells was quantified for each condition. Treatment with a physiological concentration of plasminogen caused a 3-fold increase in the number of lipid droplet–containing bone marrow–derived macrophages (Figure 1D). When the effects of plasminogen on foam cell formation in human monocyte–derived macrophages (huMDMs) were examined, a 2-fold increase in the number of lipid droplet–containing cells was observed (Figure 1 in the online-only Data Supplement).

Treatment with plasmin can lead to induction of proinflammatory cytokines that may cause cell death. To investigate whether the increase in foam cell formation observed with the addition of plasminogen was partially a result of macrophage phagocytosis of dead cells, we performed a control in which cells were incubated with plasminogen and serum-derived plasmin to accelerate foam cell formation and atherosclerosis. A detailed understanding of the mechanisms of foam cell formation is imperative for successful therapeutic targeting of atherosclerosis.
the same experiment was performed in serum-free medium, there was no statistically significant difference in foam cell formation between cells treated with agLDL alone and cells treated with plasminogen and $\alpha_2$-antiplasmin in addition to the agLDL (data not shown).

**Macrophage Incubation With agLDL Results in Increased Surface uPAR Expression and PA Activity**

To understand the mechanism by which plasminogen promotes foam cell formation, we first investigated the level of local plasmin activity at the macrophage plasma membrane. It is known that uPAR expression can be induced by fatty acid macrophage loading. Thus, we wondered whether macrophage incubation with agLDL would cause an increase in surface uPAR expression. To test this, we performed immunofluorescence surface labeling of uPAR in nonpermeabilized J774 cells incubated with agLDL. In resting cells, a small amount of uPAR staining was observed at the cell surface (Figure 2A). On treatment with agLDL for 4 hours, the amount of surface uPAR staining was significantly increased (Figure 2B). Quantification of the surface levels of uPAR at different time points revealed a sharp increase in surface uPAR levels at 2 hours that reached a plateau at 4 hours (Figure 2C). These results fit with the observation of increased uPAR staining in colocalization with macrophages in human atherosclerotic lesions.

Next, we measured cell-surface–associated PA activity as a function of agLDL incubation time. J774 macrophages were incubated with agLDL for various times followed by the addition of plasminogen for 30 minutes, and the concentration of plasmin generated was measured using a plasmin-specific chromogenic substrate. The increase in PA activity resulting from agLDL incubation was determined by the ratio of the amount of plasmin generated by macrophages incubated with agLDL divided by the plasmin concentration generated by macrophages incubated in media alone (Figure 2D). Surface PA activity increased 2.5-fold after a 4-hour incubation with agLDL, indicating that macrophages are able to generate high local levels of plasmin at the plasma membrane in response to contact with agLDL.

**Plasmin Causes Morphological Changes in the Lysosomal Synapse That Result in Compartment Tightening**

To visualize the consequences of these high local levels of plasmin, we performed time-lapse imaging of cells interacting with Alexa488-agLDL before and during plasmin treatment (Figure 3A and 3B). Cells were incubated with Alexa488-agLDL for 1 hour to allow lysosomal synapse formation followed by the addition of 1 U/mL plasmin to the sample on the microscope stage. Consistent with previous studies, time-lapse imaging shows cleavage and removal of much of the extracellular agLDL on plasmin treatment, whereas a portion of the aggregate remains cell associated (Figure 3B, see also Movie I in the online-only Data Supplement). To further characterize the fraction of the aggregate that remains cell associated after plasmin treatment, cells were incubated with colloidal gold-labeled agLDL and imaged by transmission electron microscopy. Electron microscopy confirmed the presence of surface-associated agLDL.

**Figure 1.** Macrophage incubation with aggregated low-density lipoprotein (agLDL) in the presence of a physiological concentration of plasminogen results in increased foam cell formation. Bone marrow–derived macrophages (BMMs) were incubated for 4 h with Alexa546-agLDL (red) in the absence (A) or presence of 2 $\mu$mol/L plasminogen (B and C), fixed, and labeled with LipidTOX Green (green). Cell incubation with agLDL in the presence of a physiological plasminogen concentration results in the formation of LipidTOX-positive lipid droplets (B, arrows). Application of $\alpha_2$-antiplasmin prevented the formation of LipidTOX-positive lipid droplets (C). D, Quantification of the percentage of LipidTOX-positive cells demonstrates a 3-fold increase in foam cell formation attributable to plasmin. ***P≤0.001 2-way ANOVA. Data compiled from 3 independent experiments. Error bars represent the standard error of the mean (SEM).
residing in compartments at or near the cell surface in the absence of plasmin treatment (Figure 3C, circled). We have shown previously that these are surface-connected, acidified, hydrolyse-containing compartments, which we have termed lysosomal synapses.27 After removal of much of the extracellular aggregate by plasmin, the remaining agLDL was in a compartment near the cell surface that seemed to be intracellular in EM thin sections (asterisks in Figure 3D).

To determine whether the plasmin-induced compartments are intracellular or extracellular, we tested the accessibility of streptavidin-conjugated agLDL to biotin before and after plasmin treatment (Figure 3E–3G). J774 macrophages incubated with streptavidin–Alexa488-agLDL were exposed to a 2-minute pulse of Alexa546–biotin to label extracellular biotin-accessible structures and then fixed. Consistent with previous studies, in the absence of plasmin treatment, the biotin–Alexa546 staining clearly showed that the aggregate was largely extracellular after the 1-hour incubation (Figure 3E).14 However, an Alexa546–biotin pulse followed by plasmin treatment results in the absence of biotin binding (Figure 3F), indicating that the aggregate in these compartments was not labeled during a brief incubation with Alexa546–biotin. An Alexa546–biotin pulse followed by plasmin treatment results in the formation of biotin-positive compartments (Figure 3G), confirming that the aggregate in these compartments was surface exposed before treatment. These data might indicate that the agLDL is in a fully sealed compartment, such as a phagosome, but we also considered the possibility that the surface-connected compartment had become nearly sealed but was still open to the extracellular space.

We have previously used plasma membrane labeling with fluorescent cholera toxin subunit B (CtB) to characterize the topological organization of the lysosomal synapse.27,28 In this experiment, the cells are not permeabilized, so the macromolecular CtB can only label glycolipids on the plasma membrane. We used this technique to compare the availability of agLDL-containing compartments to CtB with and without plasmin treatment (Figure 3H–3K). In contrast to CtB-positive compartments in nontreated cells (Figure 3H and 3I, arrows), agLDL-containing plasmin-induced compartments generally lack CtB labeling (Figure 3J and 3K, arrows). Plasmin-induced compartments in huMDMs also lack CtB labeling (data not shown). The ability of plasmin-induced compartments to exclude CtB and Alexa546–biotin suggests significant changes in their permeability to the extracellular space in comparison with compartments in nontreated cells. In light of these results, it seemed that plasmin treatment caused compartments to fully seal and become intracellular. Thus, we were surprised that in high-magnification 3-dimensional confocal reconstruction, agLDL in plasmin-induced CtB-negative compartments (Figure 3K, arrows) appears continuous with extracellular portions of the aggregate (Figure 3K′, arrow). The fact that agLDL in plasmin-induced CtB-negative compartments is still connected to extracellular remnants of the aggregate shows that plasmin-induced compartments preserve connection to the extracellular space.

To further investigate the surface connectivity of plasmin-induced compartments, we used a method based on pH-dependent quenching of extracellular fluorophores. This approach has been used previously to examine compartments that exclude large molecules yet preserve connection to the cell surface.35,36 For instance, endothelial cells form a subjunctional compartment during monocyte diapedesis that is not accessible to fluorescently labeled dextran but is sensitive to changes in extracellular pH.36 To examine the surface connectivity of plasmin-induced compartments via modulation of extracellular pH, J774 macrophages and huMDMs were incubated with

---

**Figure 2.** Surface urokinase-type plasminogen activator receptor (uPAR) expression and plasminogen activator (PA) activity increase during macrophage incubation with aggregated low-density lipoprotein (agLDL). J774 cells were incubated with agLDL for 0 (A) or 4 h (B), fixed without permeabilization and labeled with an antibody against uPAR. C, Quantification of surface uPAR staining of J774 cells incubated with agLDL for indicated periods of time. Values plotted are the integrated surface uPAR staining intensity per cell. Data shown are from a representative experiment from 3 independent experiments. Error bars represent the SEM. D, J774 cells were incubated in the absence or presence of agLDL for the indicated time periods followed by a 30-min incubation with 2 μmol/L plasminogen and the concentration of plasmin generated was measured using a plasmin-specific chromogenic substrate. Changes in PA activity resulting from agLDL incubation were quantified by dividing the plasmin concentration generated by cells incubated with agLDL by the plasmin concentration measured for cells incubated in media alone for the same amount of time. Data compiled from 3 independent experiments. Error bars represent the SEM.
Figure 3. Plasmin causes morphological changes in the lysosomal synapse that result in compartment tightening. A and B, Still frames from a time-lapse movie of cells before and after plasmin treatment. J774 cells were incubated with Alexa488-aggregated low-density lipoprotein (agLDL; green) for 60 min (A), 1 U/mL plasmin was added on the microscope stage and data acquisition was continued. B, Cells 30 min after plasmin treatment. J774 cells were incubated with colloidal gold-labeled agLDL for 45 min, left untreated (C) or treated with 1 U/mL plasmin during the last 15 min of incubation (D). Without plasmin treatment gold-agLDL resides in lysosomal synapses (C, circled). Plasmin treatment results in the formation of intracytoplasmic compartments containing condensed agLDL (D, asterisks). J774 cells were incubated with streptavidin-Alexa488-agLDL (green) for 60 min and left untreated (E) or treated with 1 U/mL plasmin during the last 15 min of incubation (F and G). Cells were pulsed for 2 min with Alexa546–biotin (red) at the end of incubation (E), after (F), or before (G) plasmin treatment. In the absence of plasmin treatment agLDL in the lysosomal synapse is available for biotin labeling (E, yellow). However, agLDL in plasminduced compartments is unavailable for biotin binding after plasmin treatment (absence of yellow in F). AgLDL in plasminduced compartments was surface exposed and available for biotin binding before plasmin treatment (G, yellow). J774 cells were incubated with Alexa546–agLDL (red) for 45 min (H and I) or for 30 min followed by a 15-min release with 1 U/mL plasmin (J and K) and labeled on ice with Alexa488-cholera toxin subunit B (CtB; green) before fixation. In the absence of plasmin treatment, agLDL residing in the lysosomal synapse is available for CtB labeling (H and I, yellow, arrows). Plasmin-induced compartments (J and K, arrows) are unavailable for CtB as indicated by the absence of green labeling around agLDL. K and K', High-magnification confocal image of a plasmind-treated cell. AgLDL in plasminduced CtB-negative compartments (K, arrows) preserves connection with extracellular agLDL (K', arrow). The red line in K indicates the position of the y–z reconstruction shown in K'. Similarly, the blue line in K' designates the position of the x–y plane shown in K. J774 cells and huMDMs (inset) were incubated with fluorescein isothiocyanate FITC-agLDL (L and M, green) for 45 min, treated with 1 U/mL plasmin for 15 min and quenched with 2-(N-morpholino)ethanesulfonic acid (MES; pH 5.5) buffer. FITC-agLDL (L) before quenching, (M) after quenching, and (N) corresponding differential interference contrast (DIC). AgLDL in plasminduced compartment is quenched by cell impermeant low pH buffer (M). O through Q, Control cells, in which the cytoplasm was loaded with 2’7’-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF), did not show a decrease in fluorescence on treatment with MES pH 5.5 (P), confirming that the treatment did not affect the pH of intracellular compartments. BCECF (O) before quenching, (P) after quenching, and (Q) corresponding DIC.

agLDL labeled with fluorescein isothiocyanate, a pH-sensitive fluorophore, for 45 minutes, treated with 1U/mL plasmin for 15 minutes and examined in the absence (Figure 3L) or presence (Figure 3M) of a cell impermeant low pH buffer. The fluorescence of fluorescein isothiocyanate-agLDL in plasminduced compartments is efficiently quenched by 2-(N-morpholino)ethanesulfonic acid (pH 5.5; Figure 3L–3N). Control cells, in which the cytoplasm was loaded with 2’7’-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, a fluorescent pH sensor, did not show a decrease in fluorescence on treatment with 2-(N-morpholino)ethanesulfonic acid pH 5.5 (Figure 3O–3Q), confirming that the treatment did not affect the pH of intracellular compartments. Data from huMDMs also show that the fluorescence of fluorescein isothiocyanate-agLDL in plasminduced compartments is efficiently quenched by 2-(N-morpholino)ethanesulfonic acid (pH 5.5), indicating that in human macrophages the lysosomal synapse remains surface connected after plasmin treatment (Figure 3L–3N, inset). Taken together these data show that plasminduced compartments preserve connection to the cell surface, but this connection is more nearly sealed than in compartments in nontreated cells.

Formation of Plasmin-Induced Compartments Is an F-Actin–Dependent Process

The lysosomal synapse is formed via F-actin–driven membrane protrusions surrounding the aggregate, and enhanced
actin polymerization is detected during the first hour of the cell-aggregate interaction.\textsuperscript{24} To test the role of actin polymerization in the formation and tightening of plasmin-induced compartments, we performed plasmin treatment on J774 macrophages and huMDMs in the presence of the F-actin disrupting drug latrunculin A (Figure 4). In the absence of latrunculin A treatment, agLDL was observed primarily in plasmin-induced compartments (Figure 4A). Consistent with the known function of the lysosomal synapse, the cholesterol-binding dye filipin indicates that these compartments contain high levels of FC (Figure 4B). However, plasmin treatment in the presence of latrunculin A removed almost all of the cell engaged agLDL and inhibited the formation of plasmin-induced compartments and the generation of FC (Figure 4D–4F). These data confirm the critical role of actin polymerization in the formation of plasmin-induced compartments.

**Plasmin-Induced Compartment Tightening Results in Increased Compartment Acidification**

We have previously reported that the lysosomal synapse functions as an extracellular hydrolytic organelle because of targeted exocytosis of lysosomes and compartment acidification by vacuolar (H\(^+\))-ATPase on the plasma membrane.\textsuperscript{27} The presence of lysosomal acid lipase and a low pH lead to extracellular hydrolysis of CEs.\textsuperscript{27,28} However, pH values observed in the lysosomal synapse are typically higher than in lysosomes and fluctuate because of diffusion of H\(^+\) ions into the extracellular space.\textsuperscript{27} To test how tightening of plasmin-induced compartments affects compartment acidification, we labeled LDL with CypHer 5E Mono N-hydroxysuccinimide ester and Alexa488. The fluorescence of CypHer 5E Mono N-hydroxysuccinimide ester increases as the pH decreases from 7 to 5,\textsuperscript{38} whereas Alexa488 is pH-independent in this range. Macrophages were incubated with dual-labeled agLDL, and the pH of the aggregate-containing compartment was determined from the ratio of CypHer 5E Mono N-hydroxysuccinimide ester to Alexa488 fluorescence as compared with values obtained in pH calibration buffers. When J774 and huMDMs interacted with the dual-labeled agLDL, regions of low pH could be seen at the contact sites (Figure 5A–5D). Measurements of the lowest pH achieved per compartment revealed that plasmin-induced compartments are significantly more acidic than their counterparts in nontreated cells (Figure 5E). The optimal activity for lysosomal acid lipase is at pH 5.5,\textsuperscript{39} which is the average value of the lowest pH recorded in plasmin-induced compartments. The pH in plasmin-induced compartments allows enhanced lysosomal acid lipase activity and thereby more efficient CE hydrolysis than in untreated compartments.

**CE Hydrolysis in Plasmin-Induced Compartments Is More Efficient, Leading to the Cellular Delivery of Large Amounts of FC**

The lower pH of plasmin-induced compartments suggests increased activity of lysosomal enzymes, in particular lysosomal acid lipase. To measure changes in agLDL-derived CE hydrolysis resulting from plasmin treatment, we fixed nontreated and plasmin-treated J774 macrophages and huMDMs and labeled them with the cholesterol-binding dye filipin (Figure 6A–6H). In nontreated cells, agLDL had the typical thread-like morphology, and the filipin signal was increased at sites of contact between aggregates and cells because of CE hydrolysis in the lysosomal synapse (Figure 6A–6D, arrows).\textsuperscript{27,28} Plasmin treatment caused a change in agLDL morphology resulting in the formation of condensed, cell-associated structures significantly enriched in FC, as reflected by bright filipin labeling (Figure 6E–6H, arrowheads).

To measure changes in agLDL-derived CE hydrolysis on plasmin treatment, J774 macrophages were incubated with agLDL reconstituted with \(^{14}\)C-labeled cholesteryl oleate, left untreated, or treated with plasmin, and the hydrolysis of CE was measured as described previously.\textsuperscript{27} We found that the fraction of cell-associated CE that was hydrolyzed to FC after plasmin treatment was 3x higher than in nontreated cells (Figure 6I). These data show that plasmin treatment of macrophages interacting with agLDL results in the delivery of large amounts of FC to the cell, which facilitates the increase in foam cell formation resulting from plasminogen treatment.

**Discussion**

A key pathogenic event in the development of atherosclerosis is the retention of lipoprotein particles in the subintima.\textsuperscript{40} These lipoprotein particles undergo oxidative modification, association with extracellular matrix proteoglycans, and aggregation.\textsuperscript{21} Macrophages attempt to clear the lipoproteins require distinctive mechanisms for their uptake. In particular, unlike monomeric LDL, the uptake of agLDL does not involve receptor-mediated endocytosis, but rather the aggregate is sequestered in deep invaginations at the cell surface, termed the lysosomal synapse.\textsuperscript{26–28} Our studies elucidate the mechanism of a novel pathway by which the plasmin/plasminogen system may act on macrophage-engaged agLDL contributing to accelerated foam cell formation and...
thereby atherogenesis. The results nicely reconcile the data of the Kruth group, showing that plasminogen releases agLDL from macrophages, with the reported atherogenicity of the plasmin/plasminogen system.

We have shown that plasmin can dramatically alter the early processing of cell-associated agLDL, its uptake by macrophages, and foam cell formation. Figure 7 shows a schematic of the proposed mechanism of plasmin action. Monomeric LDL in the blood stream is deposited in the subintimal space, where it becomes oxidatively modified, retained, and aggregated. When macrophages come into contact with agLDL, they form an extracellular acidic hydrolytic compartment, a lysosomal synapse (Figure 7A and 7B). Interaction of macrophages with agLDL causes upregulation of uPAR and increased PA activity, resulting in the generation of local levels of plasmin sufficient to cleave the aggregate (Figure 7C). Upregulation of macrophage uPAR is also observed in human atherosclerotic lesions, possibly because of macrophage interaction with agLDL. The aggregate located in acidic portions of the compartment probably is not cleaved further by plasmin because of the enzyme’s low activity at acidic pH.

Cleavage of macrophage-engaged agLDL results in tightening of the plasmin-induced compartment in an actin-dependent manner. Compartment tightening allows more efficient generation of an acidic environment and enhanced activity of lysosomal enzymes that are exocytosed to the lysosomal synapse (Figure 7D). This results in accelerated agLDL catabolism, leading to the delivery of large amounts of FC to the macrophage, thereby promoting foam cell formation. For clarity, we note that the lysosomal synapse forms independently of plasmin and is then modified by plasmin.

One aspect of the plasmin-induced changes in the physical organization of the lysosomal synapse that we find intriguing is the fact that the compartments do not fully seal. A proposed rationale for the need for extracellular hydrolysis in a lysosomal synapse was that macrophages could not internalize large species or moieties tightly linked to the extracellular matrix, even by phagocytosis. However, after plasmin treatment, there is no apparent reason that the compartments do not fully seal. Further, in the absence of plasmin we have repeatedly observed formation of the lysosomal synapse even with agLDL particles that are small enough to be internalized.
via phagocytosis. There is some precedence for macrophage surface-connected compartments containing lipoproteins that do not immediately seal. Macrophages sequester large β-very LDL proteins in peripheral tubular compartments that remain surface connected for several minutes. One possible reason the compartments do not fully close is that moieties generated during the catabolism of agLDL inhibit compartment sealing. Phagosome sealing occurs through contractile activities regulated by Rho-family GTPases. In particular, Rac and Cdc42 are transiently activated during phagocytosis. Rac and/or Cdc42 are also involved in macrophage agLDL interactions inducing local actin polymerization to form the lysosomal synapse. We have previously reported that loading of macrophages with FC causes increased Rac activity. Interestingly, it was shown that constant activation of Rac causes a delay in phagosome closure. Thus, it is possible that agLDL-derived FC and potentially other moieties generated during the catabolism of agLDL affect Rho-family GTPase activities in a way that interferes with their activation–inactivation cycle, thus preventing compartment sealing. The detailed signaling mechanisms responsible for lysosomal synapse formation and function are currently under investigation.

The fact that the plasmin-induced compartments retain connection to the extracellular space is relevant to both atherogenesis and macrophage biology. Pharmacological approaches to inhibit lipid accumulation by macrophage foam cells have been pursued intensely as they are thought to be of value in preventing coronary artery disease. However, successes have been limited, possibly because of an incomplete understanding of the mechanisms of foam cell formation in vivo. The cellular mechanisms for agLDL catabolism are likely to be significantly different for extracellular hydrolysis by lysosomal enzymes as opposed to degradation within a phagolysosome. As a consequence, different sets of soluble N-ethylmaleimide–sensitive factor attachment protein receptors and other proteins are used for lysosome fusion with phagosomes and lysosome fusion with the plasma membrane. To develop efficacious therapeutic approaches, the exact mechanism by which macrophages become foam cells must be understood.

Further, the extracellular catabolism of agLDL may play a role in the development of the atherosclerotic core itself. We have shown in our previous work that lysosomal synapses are dynamic. They can be tightly sealed at times, but they also transiently open up, a process that would not happen with a fully sealed phagolysosome. This opening can release catabolic products, such as unesterified cholesterol, into the extracellular space. Significant increases in the ratio of free to total cholesterol have been observed during progression of plaques from fatty streaks to fibrolipid lesions. Extracellular FC is present in excess in advanced plaques, particularly those prone to rupture and, therefore, may represent an underlying sign of lesion instability. However, the source of this unesterified cholesterol is currently unknown.
also play a role in the formation of extracellular cholesterol crystals, implicated as a mechanical factor that contributes directly to plaque vulnerability. Several additional enzymes are likely released into the extracellular space during the process of macrophage agLDL catabolism, including lysosomal hydrolases, which have been implicated in some studies to increase fusion of lipoproteins and hydrolyze the extracellular matrix.

In conclusion, the data presented herein indicate that in addition to remodeling the extracellular matrix, plasmin may be involved in lipoprotein modification and foam cell formation during genesis and progression of atherosclerotic lesions. Plasmin-induced macrophage cholesterol accumulation is a novel pathway by which the plasmin/plasminogen system may contribute to atherogenesis. These findings provide new insight into the atherogenicity of plasmin and implicate a specific mechanism by which plasmin can accelerate atherosclerosis. Elucidation of this pathway may enable the development of novel, targeted therapies for the prevention of atherosclerosis. This newly identified pathway for the plasmin/plasminogen system to regulate foam cell formation provides a new context in which to understand the mechanism by which plasmin plays a role in proatherogenic processes.

Figure 7. Proposed model explaining the mechanism of plasmin-induced foam cell formation. A, Monomeric low-density lipoprotein (LDL; red) in the blood stream is deposited in the subintimal space, where it becomes oxidatively modified, retained, and aggregated. When subintimal macrophages (purple) interact with aggregated LDL (agLDL; red), an extracellular, acidic, hydrolytic compartment, a lysosomal synapse, is created. B, The lysosomal synapse is formed by F-actin (green)-driven plasma membrane protrusions. The low pH of the compartment is maintained by V-ATPase in the macrophage plasma membrane. Free cholesterol (FC) may be transferred to the macrophage plasma membrane after hydrolysis of LDL cholesteryl esters (CEs) by lysosomal acid lipase that has been delivered to the compartment via lysosome exocytosis. C, Macrophage interaction with agLDL results in upregulation of surface urokinase-type plasminogen activator receptor (uPAR) expression and increased plasminogen activator (PA) activity resulting in the generation of local levels of plasmin sufficient to cleave the aggregate. The aggregate located in acidic portions of the compartment is not cleaved by plasmin because of its low activity at acidic pH. D, Proteolysis of macrophage-engaged agLDL results in tightening of the plasmin-induced compartment in an actin-dependent process. Compartment tightening allows efficient generation of an acidic environment and enhanced activity of lysosomal enzymes which are exocytosed to the lysosomal synapse. This results in accelerated agLDL catabolism leading to the delivery of large amounts of FC to the macrophage and thereby promoting foam cell formation.
References


Acknowledgments

We thank Leona Cohen-Gould for electron microscopy analysis and Haley Fraser for figure editing.

Sources of Funding

This work was supported by National Institutes of Health grants R37-DK27083 and R01-HL093324 to F.R. Maxfield.

Disclosures

A.S. Haka was supported by National Research Service Award Fellowship from the National Institutes of Health. The other authors report no conflicts.
The plasmin/plasminogen system is involved in atherosclerosis. However, the mechanisms by which it stimulates disease are not fully defined. In atherogenesis, low-density lipoprotein (LDL) is deposited on arterial walls where it is modified, aggregated, and retained. Macrophages are recruited to clear the LDL, and they become foam cells. This study assesses the role of plasmin in macrophage uptake of aggregated LDL. We found that plasminogen treatment of macrophages catalyzes aggregating LDL accelerated foam cell formation. This occurs because plasmin proteolyses cell-associated aggregated LDL, allowing a portion of the aggregate to become sequestered in a nearly sealed, yet extracellular, acidic compartment. The low pH in the plasmin-induced compartment allows lysosomal enzymes, delivered via lysosome exocytosis, greater activity, resulting in more efficient cholesteryl ester hydrolysis and delivery of a large cholesterol load to the macrophage, thereby promoting foam cell formation. These findings provide a new context for considering the atherogenic role of plasmin.
Plasmin Promotes Foam Cell Formation by Increasing Macrophage Catabolism of Aggregated Low-Density Lipoprotein
Abigail S. Haka, Inna Grosheva, Rajesh K. Singh and Frederick R. Maxfield

Arterioscler Thromb Vasc Biol. 2013;33:1768-1778; originally published online May 23, 2013; doi: 10.1161/ATVBAHA.112.301109
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/33/8/1768

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2013/05/23/ATVBAHA.112.301109.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
MATERIALS AND METHODS

Cells and Cell Culture. J774a.1 macrophage-like cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 units/ml penicillin and 50 µg/ml streptomycin in a humidified atmosphere (5% CO₂) at 37°C. Bone marrow derived macrophages (BMMs) isolated from C57BL/6 mice were differentiated for 7 days by culture in the same media supplemented with 20% L-929 cell conditioned media. Human monocytes (Life Line Cell Technology, Frederick, MD) were differentiated into macrophages in vitro by incubation in RPMI containing 10% heat-inactivated FBS and 10 ng/ml macrophage colony stimulating factor (R&D Systems, Minneapolis, MN) for 5 days. For all live cell imaging experiments, media was changed to DMEM containing 25 mM 4-(2-hydroxyethyl)-1-pipierazine ethane sulphonate acid without phenol red or sodium bicarbonate.

Lipoproteins and Reagents. Human LDL was prepared from donor plasma as described¹. LDL was labeled using succinimidyl esters of AlexaFluor-546 (Alexa546) and Alexa488 (Invitrogen, Carlsbad, CA), fluorescein isothiocyanate (FITC), biotin (Sigma-Aldrich, St. Louis, MO) or CyphHer 5E Mono N-hydroxysuccinimide ester (CyphHer5E) (GE Healthcare, Chalfont St. Giles, U.K.). LDL was aggregated by vigorous vortexing for 10 sec². Alexa546-biocytin, Alexa488-cholera toxin subunit B (CtB), LipidTOX Green, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxy-methyl ester (BCECF-AM) and latrunculin A (LatA) were purchased from Invitrogen. Filipin, 2-(N-mopholino)ethanesulfonic acid (MES), streptavidin and acyl-coenzyme A cholesterol acyltransferase (ACAT) inhibitor 58035, were purchased from Sigma-Aldrich.

Plasmin and Plasminogen Treatment. Plasmin from human plasma was purchased from Sigma-Aldrich. Cells were incubated for the indicated time periods with aggregated LDL (agLDL) to allow attachment and formation of the lysosomal synapse, followed by treatment with 1 U/ml plasmin for 15 min. Plasminogen and α₂-antiplasmin were purchased from Calbiochem (Darmstadt, Germany). Plasminogen was tested for the absence of residual plasmin activity with a chromogenic substrate. To assay for foam cell formation, BMMs were incubated with agLDL for 4 hr in presence or absence of 2 µM plasminogen with or without 150 µg α₂-antiplasmin.

Plasminogen activator activity measurements. J774 cells were incubated with or without agLDL for indicated time periods in phenol red free DMEM containing 10% FBS. After incubation, cells were rinsed with serum-free medium and incubated in serum-free medium containing 2 µM plasminogen for 30 min. Medium was centrifuged at 14000 rpm for 5 min to remove cell debris and unattached agLDL. Plasmin activity in supernatants was assessed by addition of chromogenic substrate CS 41(03) (Aniara, West Chester, OH) according to the manufacturer’s protocol, and the reaction stopped after 5 min by addition of 5% (w/v) citric acid. Absorbance was measured at 405 nm using a SpectraMAX M2 spectrophotometer (Molecular Devices, Sunnyvale, CA). To calculate relative increases in plasminogen activator activity, the optical density at 405 nm measured in medium conditioned by cells incubated with agLDL was divided by the optical density at 405 nm measured in medium conditioned by cells incubated without agLDL for the same time period.

Cell labeling and Microscopy. Labeling with LipidTOX Green was performed according to the manufacturer’s protocol. To determine surface urokinase-type plasminogen activator receptor (uPAR) expression, J774 cells were incubated with agLDL for various times, fixed without permeabilization in 3% paraformaldehyde (PFA) and labeled with an antibody against uPAR at 1:50 dilution for 60 min (rabbit polyclonal against full length uPAR, FL-290, Santa Cruz Biotechnology, Santa Cruz, CA). Cell labeling with Alexa488-CtB and filipin and was performed as described³. Labeling of extracellular streptavidin-
conjugated agLDL was accomplished with a 2 min pulse of 200 µM Alexa546-biocytin. For cytoplasmic loading with pH sensitive dye, J774 cells were incubated with 40 µM BCECF-AM in medium 2 and 0.2%(w/v) glucose containing 250 µM sulfinpyrazone for 1 hr. For actin de-polymerization, J774 cells were incubated with agLDL for 45 min without plasmin or incubated with agLDL for 30 min and then treated with 1 U/ml plasmin during the last 15 min of aggregate incubation. 5 µM LatA was added during the last 15 min of incubation to both plasmin treated and untreated cells.

For imaging, cells were plated on Poly-D-lysine coated glass-coverslip bottom dishes. Images were acquired with a Leica DMIRB widefield microscope equipped with a 40x, 1.25 numerical aperture (NA) plan Apochromat objective, or a Zeiss LSM510 laser scanning confocal microscope using a 63x, 1.4 NA plan Apochromat objective. For time-lapse data sets, a 40x 0.8 NA objective was employed, and pinholes on the confocal microscope were opened (so laser power could be lowered to reduce photobleaching), resulting in an axial resolution of 14 µm. Cell temperature was maintained at 37°C with a heated stage and objective heater. Where indicated, plasmin was added to dishes on the microscope stage.

Electron Microscopy. J774 cells were incubated with colloidal gold-labeled agLDL for 1 hr with or without the addition of plasmin for the last 15 min. Following incubation with agLDL the cells were fixed with a modified Karnovsky’s solution containing 2.5% glutaraldehyde, 4% PFA and 0.02% picric acid, postfixed with 1% Osmium tetroxide, 1.5% Potassium ferricyanide, treated with uranyl acetate, dehydrated through a graded ethanol series and embedded in LX112 resin. En face serial sections were cut at 70 nm thickness and picked up on formvar-coated, 4-slot copper grids. Sections were further contrasted with uranyl acetate and lead citrate. Images were acquired at Weill Cornell Medical College on a JEOL JEM 100CX-II electron microscope operating at 80kV at a set magnification of 10,000X.

pH Measurements. J774 macrophages were incubated for 60 min with CypHer 5E (a pH sensitive fluorophore) and Alexa488 (a pH insensitive fluorophore) dual labeled agLDL. The pH value within each pixel was assessed quantitatively by comparison with ratio images obtained in calibration buffers of varying pH as described previously. Live cells were imaged on the confocal microscope using a 63x 1.4 NA objective.

All data were analyzed with MetaMorph image analysis software, Molecular Devices Corporation (Downington, PA). A binary mask was created using the Alexa488 signal intensity and applied to both channels to remove background noise. Images were convolved with a 7x7 pixel Gaussian filter, and ratio images were generated.

Radiolabeled CE hydrolysis Measurement. LDL was reconstituted with cholesteryl-[4-14C]-oleate (American Radiolabeled Chemicals, St. Louis, MO) as described. Cells were incubated with radiolabeled reconstituted agLDL for 90 min in the presence of 30 µg/ml ACAT inhibitor (to prevent re-esterification of hydrolyzed [14C]cholesteryl ester), left untreated or treated with plasmin for the last 15 min of incubation. Cell associated lipids were extracted, and the amount of cholesteryl ester and free cholesterol was quantified as described.

Statistics. Statistical analysis was performed using Excel. For comparisons of two groups, student’s t test was used. For comparisons of more than two groups, two-way ANOVA was used.
SUPPLEMENTARY MATERIAL REFERENCES


SUPPLEMENTAL MATERIAL

SUPPLEMENTAL FIGURES

Figure I. Macrophage incubation with agLDL in the presence of a physiological concentration of plasminogen results in increased foam cell formation. huMDMs were incubated for 4 hr with Alexa546-agLDL in the absence or presence of 2 µM plasminogen, fixed and labeled with LipidTOX Green. Quantification of the percentage of LipidTOX-positive cells demonstrates a two-fold increase in foam cell formation attributable to plasmin. ** P ≤ 0.01, *** P ≤ 0.001 two-way ANOVA. Data compiled from one experiment. Error bars represent the SEM.

Movie I. Plasmin mediated cleavage of macrophage associated agLDL. J774 cells were incubated with Alexa488-agLDL (green) for 60 min prior to data acquisition. Cells were imaged for 7.5 min and then 1 U/ml plasmin was added on the microscope stage. Data acquisition was continued for 112.5 min.