Plasmin-mediated Macrophage Reversal of Low Density Lipoprotein Aggregation*

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Evidence suggests that aggregated low density lipoprotein (AgLDL) accumulates in atherosclerotic lesions. Previously, we showed that AgLDL induces and enters surface-connected compartments (SCC) in human monocyte-derived macrophages by a process we have named patocytosis. Most AgLDL taken up by these macrophages in the absence of serum is stored in SCC and remains undegraded. We now show that macrophages released AgLDL (prepared by vortexing or treatment with phospholipase C or sphingomyelinase) from their SCC when exposed to 10% human lipoprotein-deficient serum (LPDS). Macrophages also took up AgLDL in the presence of LPDS, but subsequently released it. In both cases, the released AgLDL was disaggregated. Although the AgLDL that macrophages took up could not pass through a 0.45-μm filter, >60% of AgLDL could pass this filter after release from the macrophages. Disaggregation of AgLDL was verified by gel-filtration chromatography and electron microscopy that also showed particles larger than LDL, reflecting fusion of LDL that aggregates. The factor in serum that mediated AgLDL release and disaggregation was plasmin generated from plasminogen by macrophage urokinase plasminogen activator. AgLDL release was decreased >90% by inhibitors of plasmin (ε-aminocaproic acid and anti-plasminogen mAb), and also by inhibitors of urokinase plasminogen activator (plasminogen activator inhibitor-1 and anti-urokinase plasminogen activator mAb).

Not all cholesterol in atherosclerotic lesions accumulates within macrophages. Much of this cholesterol accumulates as cholesteryl ester-rich lipid particles in the extracellular spaces of atherosclerotic lesions, and these particles are particularly enriched in the lipid-rich core of lesions. Evidence suggests that the cholesteryl ester-rich lipid particles are derived from LDL rather than from cellular lipid droplets (reviewed in Ref. 18). The particles resemble LDL in having linoleate as the major cholesteryl ester fatty acyl group, while cellular lipid droplets have oleate as their major cholesteryl ester fatty acyl group. One important difference between extracellular cholesteryl ester-rich particles and LDL or cellular lipid droplets is that while LDL are 22 nm in diameter and cellular lipid droplets are >400 nm, the cholesteryl ester-rich lipid particles range 40 to 200 nm in diameter.

Many modifications to LDL have been shown to cause LDL to aggregate (3, 8, 9, 19). These include oxidation of LDL and treatment of LDL with certain lipases that are present within lesions (sphingomyelinase, phospholipases A2 and C). Simple vortexing of LDL is a convenient way to produce aggregated LDL (AgLDL) (10). Most of these modifications not only cause LDL to aggregate but also to undergo fusion (20–23). Fusion of the LDL particles causes them to attain sizes similar to the cholesteryl ester-rich lipid particles that accumulate in the extracellular spaces of atherosclerotic lesions. Electron microscopy shows that these LDL-like cholesteryl ester-rich lipid particles can occur as individual particles in lesions (24). Also, lipoprotein-deficient serum; PAI-1, plasminogen activator inhibitor-1.

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1 The abbreviations used are: LDL, low density lipoprotein; AgLDL, aggregated LDL; SCC, surface-connected compartments; LPDS, human lipoprotein-deficient serum; PAI-1, plasminogen activator inhibitor-1.

2 W.-Y. Zhang and H. S. Kruth, unpublished data.
the lipid particles can be isolated from lesions as individual non-aggregated particles (18).

Currently, no model links the occurrence of fused LDL in aggregates with the presence of lesion extracellular cholesteryl ester-rich lipid particles. Here, we report that upon exposure to and activation of plasminogen, macrophages disaggregate and release AgLDL that these cells have accumulated in SCC. Disaggregation of aggregated and fused LDL is one possible mechanism by which LDL could form cholesteryl ester-rich lipid particles larger than LDL, and resembling those lipid particles that accumulate within the extracellular spaces of atherosclerotic lesions.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human LDL (RP-032) and human lipoprotein-deficient serum (LPDS) (RP-052) were obtained from Intracel; human 125I-LDL (BT-913R) from Biomedical Technologies; bovine lung aprotinin (194559) from ICN; human plasminogen (528179), human plasmin (527624), human recombinant plasminogen activator inhibitor-1 (528205), e-amino caproic acid (1381), and cytochalasin D (250255) from Calbiochem; lysine-agarose (L9268), spgomyelina (57651), phospholipase C (P9439), chloroquine (C6626), and mouse myeloma protein MOPC21 (M9269) were from Sigma; RPMI 1640 medium from Life Technologies, Inc.; mouse anti-plasminogen monoclonal antibody (Ab-10-V1) from Research Diagnostics; mouse monoclonal antibodies polyclonal against human urokinase plasminogen activator (3940A) and human tissue plasminogen activator (374B) from American Diagnostics; human TIMP-I (CC1062) from Chemicon; human TIMP-II (178294) and phosphoramidon (874531) from Roche Molecular Biochemicals; anti-LDL receptor mouse monoclonal antibody (C7) was purified from supernatant of hybridoma CRL 1691 obtained from American Type Culture Collection.

Preparation of Aggregated Lipoproteins—LDL was aggregated by vortexing as described in Ref. 10 or was aggregated with phospholipase C and spgomyelina essentially as described before (9, 20, 21). Filtration of vortexed LDL through a 0.45-μm filter eliminated macrophage uptake and degradation of 125I-AgLDL. This showed that only the aggregated fraction of LDL could induce and enter macrophage SCC. Therefore, only the aggregated fraction of LDL isolated by centrifugation at 14,000 × g for 15 min, was incubated with macrophages. Except where stated otherwise, experiments were carried out with AgLDL produced by vortexing.

**Cell Culture and Assays**—Preparation of human fibroblast and 2-week-old human monocyte-derived macrophage cultures and assays of lipoprotein metabolism and cholesterol content of cells were carried out as described previously (9, 20, 21). All data are presented as mean ± S.E. determined from 3 culture wells for each data point. No error bar is shown when the error was smaller than the symbol height.

Preparation of Macrophage Disaggregated AgLDL and Incubation with Cells—Macrophages were incubated 24 h with 50 μg/ml 125I-AgLDL in the presence of RPMI 1640 medium with 10% LPDS, then, media were collected, pooled, and dialyzed against a 200-fold greater volume of RPMI 1640 to remove most trichloroacetic acid-soluble 125I-tyrosine produced during the initial incubation of 125I-AgLDL with macrophages. Next, the pooled media were filtered (0.45-μm pore size) to produce a fraction that contained 8.5–16 μg/ml disaggregated trichloroacetic acid-insoluble 125I-AgLDL. Metabolism of the disaggregated trichloroacetic acid-insoluble 125I-AgLDL was assessed by incubating this medium with fibroblasts and fresh macrophages. Control incubations were carried out by incubating fibroblasts and fresh macrophages with similar amounts of 125I-LDL added to medium conditioned by macrophages 24 h without 125I-AgLDL.

**RESULTS**

Release of AgLDL from Macrophage SCC Was Mediated by Plasmin Derived from Serum—After macrophages accumulated 125I-AgLDL within SCC (for either 5 or 24 h) and were then exposed to LPDS for 24 h, macrophages released much of their accumulated 125I-AgLDL back into the culture medium. Most 125I-AgLDL was released as trichloroacetic acid-insoluble material indicating the presence of relatively intact LDL protein (Table I). This occurred for LDL aggregated by vortexing (Fig. 1A) and also for LDL aggregated by exposure to phospholipase C or spgomyelina (Fig. 2). Electron microscopy confirmed loss of AgLDL from macrophage SCC upon exposure of macrophages to LPDS (Fig. 3).

Previously, we had shown that exposure of macrophages to trypsin could release AgLDL from SCC (15). Therefore, we tested whether the factor in LPDS that released AgLDL was a protease. The serum factor was sensitive to aprotinin, a serine protease inhibitor (Figs. 1A and 2), suggesting that serum contained some serine protease activity that induced release of AgLDL from macrophage SCC. Moreover, this protease could function in the presence of the many naturally occurring protease inhibitors that serum contains.

Plasmin is a serum serine protease that shows resistance to serum protease inhibitors when it is bound to the macrophage cell surface (28). Serum plasminogen is converted to active plasmin by urokinase and tissue plasminogen activators both of which are produced by macrophages within atherosclerotic lesions (29–34). Many findings indicated that plasmin was the serum factor that mediated release of AgLDL from macrophage SCC. First, the releasing activity of LPDS was decreased 96 ± 10% when LPDS was adsorbed with lysine-agarose, an agent that binds plasminogen and plasmin. Second, e-amino caproic acid (25 mM), a plasmin inhibitor, decreased by 100 ± 17% LPDS-induced release of 125I-AgLDL from macrophages. Last, an anti-plasminogen/plasmin monoclonal antibody added to LPDS decreased by 96 ± 10% LPDS-induced release of 125I-AgLDL (Fig. 1B). Plasmin is reported to activate matrix metalloproteinases released by macrophages (35). However, we found no evidence that macrophage-derived matrix metalloproteinases were required for plasmin-mediated release of macrophage-association 125I-AgLDL TIMP-I (1 μg/ml), TIMP-II (1 μg/ml), and phosphoramidon (150 μg/ml) inhibitors of matrix

### Table I

| Preincubation       | Chase      | 125I-AgLDL distribution after chase |
|---------------------|------------|------------------------------------|
|                     |            | Cell-associated | Medium trichloroacetic acid-soluble | Medium trichloroacetic acid-insoluble |
| 125I-AgLDL, 5 h     | No addition, 24 h | 85.1 ± 0.6 | 11.1 ± 0.5 | 3.8 ± 0.5 |
| 125I-AgLDL, 5 h     | No addition, 24 h | 80.0 ± 1.3 | 17.0 ± 1.1 | 3.0 ± 0.2 |
| 125I-AgLDL, 5 h     | LPDS, 24 h  | 9.7 ± 0.3  | 28.3 ± 0.7 | 62.0 ± 0.6 |
| 125I-AgLDL, 5 h     | Plasminogen, 24 h | 8.2 ± 0.5 | 45.8 ± 0.7 | 46.0 ± 1.2 |
| None               | 125I-AgLDL, 24 h | 82.5 ± 2.3 | 3.2 ± 0.3 | 14.3 ± 2.5 |
| None               | 125I-AgLDL + LPDS, 24 h | 10.7 ± 0.7 | 21.5 ± 0.8 | 67.8 ± 1.4 |

*a* Data from Fig. 1.  
*b* Data from Fig. 5 except for medium trichloroacetic acid-insoluble data that is not shown in Fig. 5 but was obtained in the same experiment. Medium trichloroacetic acid-insoluble data following filtration is shown in Fig. 5, and medium trichloroacetic acid-insoluble data obtained from unfiltered samples is shown here for comparison with other similar data in the table.
metalloproteinases did not inhibit LPDS (or purified plasminogen)-stimulated release of 125I-AgLDL from macrophages.

Macrophage Conversion of Serum Plasminogen to Active Plasmin Was Necessary for Release of AgLDL from Macrophages—The plasmin activity that mediated release of 125I-AgLDL from human monocyte-macrophages was generated from serum plasminogen by the macrophages. Macrophages that had accumulated 125I-AgLDL during a 5-h incubation with 50 μg/ml 125I-AgLDL, released 60% of their cell-associated 125I-AgLDL when exposed to trypsin (50 μg/ml) for 30 min, but released none of their 125I-AgLDL when exposed to 10% LPDS for this short period. The fact that LPDS releasing activity was present after 24 h (Fig. 1A) but not after 30 min of incubation is consistent with a time-dependent generation of a releasing factor in LPDS. Addition of the natural inhibitor of plasminogen activator, plasminogen activator inhibitor-1 (PAI-1) (16.5 μg/ml) to LPDS diminished LPDS releasing activity by 91 ± 11%. Also, a purified mouse monoclonal antibody that inhibits activity of urokinase plasminogen activator (200 μg/ml), decreased LPDS releasing activity by ~99%. In contrast, a purified mouse monoclonal antibody that inhibits tissue plasminogen activator did not affect LPDS releasing activity. Last, the releasing activity of LPDS could be replaced by substituting purified plasminogen for LPDS (Fig. 1C and Table I). LPDS and purified plasminogen not only caused macrophage release of trichloroacetic acid-insoluble 125I-AgLDL protein, but these agents also caused macrophage release of LDL cholesterol (Table II). This showed that LDL particles and not just their protein components were released from macrophages.

Macrophage Reversal of LDL Aggregation

Macrophage-generated Plasmin Caused Some Degradation of AgLDL—Besides inducing release of macrophage accumulated 125I-AgLDL, LPDS also increased degradation of 125I-AgLDL. Previously, we showed that following 125I-AgLDL entry into macrophage SCC, chloroquine-sensitive (presumably lysosomal) degradation of some 125I-AgLDL occurred (15). However, the increase in 125I-AgLDL degradation stimulated by exposure of macrophages to LPDS or plasminogen was not mediated by macrophage lysosomes because chloroquine (100 μM) did not inhibit this degradation. The degradation was due to macrophage-generated plasmin because inhibition of plasmin with 25
Greater than 60% of the released $^{125}\text{I}-\text{AgLDL}$ passed through a exposure to LPDS or plasminogen was much less aggregated. (14). However, the $^{125}\text{I}-\text{AgLDL}$ released from SCC following aggregation—Only $\text{AgLDL}$ induced and entered macrophage SCC showing that plasmin could degrade the protein component (i.e. apoB) of LDL as previously reported (36). Macrophage-generated Plasmin Caused Reversal of LDL Aggregation—Only $\text{AgLDL}$ induced and entered macrophage SCC (14). However, the $^{125}\text{I}-\text{AgLDL}$ released from SCC following exposure to LPDS or plasminogen was much less aggregated. Greater than 60% of the released $^{125}\text{I}-\text{AgLDL}$ passed through a 0.45-μm (pore size) filter (Table III, part A), while less than 10% of the original $^{125}\text{I}-\text{AgLDL}$ passed through the 0.45-μm filter. Direct exposure of $^{125}\text{I}-\text{AgLDL}$ (50 μg/ml) to plasmin (but not plasminogen) for 1 day converted >84% of the $^{125}\text{I}-\text{AgLDL}$ (both vortexed or lipase-treated) to a filtrable form. This showed that plasmin was sufficient to disaggregate $^{125}\text{I}-\text{AgLDL}$. The size distribution of macrophage-disaggregated $\text{AgLDL}$ (the fraction that passed through a 0.45-μm filter) was compared with LDL by gel-filtration chromatography (Fig. 4). It was not possible to assess the size of the initial $\text{AgLDL}$ added to macrophage cultures because it was too large to enter the gel-filtration column. Macrophage disaggregation of $\text{AgLDL}$ generated lipid particles that eluted both within the elution range (fractions 30–60) of LDL (62% of eluted disaggregated $\text{AgLDL}$ cholesterol) and earlier than the LDL elution range (38% of eluted disaggregated $\text{AgLDL}$ cholesterol).

Incubation of $^{125}\text{I}-\text{AgLDL}$ with macrophages in the presence of LPDS as a source of plasminogen did not prevent initial macrophage uptake of $^{125}\text{I}-\text{AgLDL}$ (Fig. 5A). However, over time the cell-associated $^{125}\text{I}-\text{AgLDL}$ decreased during incubation with LPDS. Simultaneously, the $^{125}\text{I}-\text{AgLDL}$ was progressively released into the medium in disaggregated form shown by the increasing amount of medium trichloroaetic acid-insoluble $^{125}\text{I}-\text{AgLDL}$ that could be filtered (Fig. 5C and Table I). This was accompanied by an increase in degradation of $^{125}\text{I}-\text{AgLDL}$ that was mostly due to plasmin activity because degradation was inhibited 73% by anti-plasminogen monoclonal antibody (Figs. 5B and 6). Degradation of monomeric $^{125}\text{I}-\text{LDL}$ (50 μg/ml) incubated 24 h with macrophages did not increase in the presence of 10% LPDS (degradation was 0.5 + 0.0 μg/mg of cell protein both with and without 10% LPDS).

Macrophages accumulated about 75% as much cell-associated $^{125}\text{I}-\text{AgLDL}$ when incubated 5 h with $^{125}\text{I}-\text{AgLDL}$ (50 μg/ml) and plasminogen (1 unit/ml) as when incubated with $^{125}\text{I}-\text{AgLDL}$ alone. However, as was the case for macrophages incubated with $^{125}\text{I}-\text{AgLDL}$ in the presence of LPDS, during prolonged incubation (24 h) of macrophages with $^{125}\text{I}-\text{AgLDL}$ and plasminogen, >90% of cell-associated $^{125}\text{I}-\text{AgLDL}$ was subsequently released. Macrophages exposed to $^{125}\text{I}-\text{AgLDL}$ for 48 h in the presence of 1 unit/ml plasminogen also disaggregated $^{125}\text{I}-\text{AgLDL}$ such that >50% of the total trichloroaetic acid-insoluble $^{125}\text{I}-\text{AgLDL}$ passed through a 0.45-μm filter. Electron microscopy of negatively stained samples of unfiltered culture media showed that $\text{AgLDL}$ was not disaggregated when exposed to plasminogen without macrophages (Fig. 7A), but was converted to individual lipid particles that ranged in size from 22 to 75 nm when exposed to plasminogen in the presence of macrophages (Fig. 7B). Similar-sized lipoprotein particles were released from macrophages that first were allowed to accumulate $\text{AgLDL}$ (100 μg/ml) for 5 h, and then were exposed to plasminogen or LPDS for 24 h to cause disaggregation and release of $\text{AgLDL}$ from macrophages.

When serum was present, interaction of $^{125}\text{I}-\text{AgLDL}$ with macrophages was required for reversal of its aggregation. Medium removed from macrophage cultures after conditioning for 24 h in the presence of 10% LPDS did not disaggregate subsequently added $^{125}\text{I}-\text{AgLDL}$ (50 μg/ml) during a 24-h incubation. This finding is consistent with the fact that serum contains plasmin inhibitors, and in the presence of serum, only macrophage-bound plasmin is active (28). When serum was absent, culture medium containing plasminogen conditioned 24 h by macrophages could disaggregate $^{125}\text{I}-\text{AgLDL}$ (72% of this treated $^{125}\text{I}-\text{AgLDL}$ passed through a 0.45-μm filter) (Table III, part B). This showed that macrophages could generate active plasmin in the culture medium but that in the presence of serum, the activity of plasmin in the culture medium was inhibited.

Effects of Macrophage Cholesterol Enrichment on Uptake, Release, and Disaggregation of $\text{AgLDL}$—Cholesterol-enriched macrophages retained their capacity to accumulate $^{125}\text{I}-\text{AgLDL}$, and subsequently disaggregate and release this $^{125}\text{I}$-

| Condition | Macrophage cholesterol content (μmol/mg cell protein) |
|-----------|-----------------------------------------------------|
| 0 days    | 75 ± 2                                              |
| $\text{AgLDL}$, 1 day | 361 ± 2                                           |
| $\text{AgLDL}$, 1 day/no addition | 359 ± 9                                         |
| 1 day     |                                                     |
| $\text{AgLDL}$, 1 day/LPDS 1 day | 209 ± 13                                          |
| $\text{AgLDL}$, 1 day/LPDS + | 351 ± 9                                          |
| aprotinin 1 day |                                              |
| $\text{AgLDL}$, 1 day/plasminogen | 230 ± 3                                           |
| 1 day     |                                                     |
| $\text{AgLDL}$, 1 day/plasminogen + aprotinin 1 day | 334 ± 7                                          |

TABLE II

Plasminogen induced release of cholesterol from macrophages

Two-week-old monocyte-macrophage cultures were incubated with 200 μg/ml $\text{AgLDL}$ in RPMI 1640 for 1 day, rinsed 3 times with RPMI 1640 medium, and incubated 1 day in RPMI 1640 medium with either plasminogen (1 unit/ml) ± aprotinin (0.15 unit/ml) or 10% LPDS ± aprotinin. Then, cultures were rinsed and analyzed for their total cholesterol content.

FIG. 4. Gel-filtration chromatographic analysis of macrophage disaggregated $\text{AgLDL}$. Two-week-old monocyte-macrophage cultures were incubated 5 h with $\text{AgLDL}$ prepared by vortexing (Vx$\text{AgLDL}$). Then, macrophage cultures were rinsed and incubated 24 h in RPMI 1640 medium with 1 unit/ml plasminogen to cause macrophage release and disaggregation of $\text{AgLDL}$. Following this incubation, media were collected, pooled, concentrated, and gel-filtered through 2% agarose (B) as described previously (25). Native LDL was eluted through the same gel for comparison (A). Fractions were assayed for cholesterol. Fraction 18 is the void volume peak.
Macrophage Reversal of LDL Aggregation

AgLDL. Macrophages were enriched with cholesterol by incubating them in RPMI 1640 medium plus 50 μg/ml acetylated LDL for 2 days. Macrophage total cholesterol content increased from 105 ± 6 to 191 ± 9 nmol/mg of cell protein. Control macrophages incubated 2 days without acetylated LDL showed a cholesterol content of 98 ± 4 nmol/mg of cell protein. Further incubation of these control and cholesterol-enriched macrophages with 50 μg/ml 125I-AgLDL for 5 h produced a similar accumulation of cell-associated 125I-AgLDL, 39.9 ± 0.9 and 40.4 ± 4.5 μg/mg of cell protein, respectively. A subsequent 24-h incubation in 10% LPDS produced about the same decrease in cell-associated 125I-AgLDL to 5.0 ± 0.7 and 3.6 ± 0.1 μg/mg of cell protein for control and cholesterol-enriched macrophages, respectively. Also, cholesterol enrichment of macrophages did not impair macrophage disaggregation of 125I-AgLDL induced by 10% LPDS. Both control and cholesterol-enriched macrophages showed about the same fold increase (6.6 and 7.5, respectively) in medium filtrable trichloroacetic acid-insoluble 125I-AgLDL over control and cholesterol-enriched macrophages incubated without 10% LPDS.

Cellular Metabolism of Macrophage-disaggregated AgLDL—Macrophage metabolism of disaggregated AgLDL was assessed. Disaggregated 125I-AgLDL was produced by incubating macrophages with 125I-AgLDL in the presence of 10% LPDS, dialyzing the collected media to remove trichloroacetic acid-soluble 125I-tyrosine, then filtering media to produce a fraction containing disaggregated trichloroacetic acid-insoluble 125I-AgLDL over control and cholesterol-enriched macrophages incubated without 10% LPDS.

Fig. 5. Time course of macrophage metabolism of AgLDL incubated in the presence of LPDS. Two-week-old monocyte-macrophage cultures were incubated up to 24 h with 50 μg/ml 125I-AgLDL in 1 ml of RPMI 1640 medium without and with 10% LPDS. At the indicated times, media were collected, macrophage monolayers were rinsed, and cell-associated 125I-AgLDL was determined (A). Then, the media content of trichloroacetic acid-soluble (B) and filtrable trichloroacetic acid-insoluble 125I-AgLDL (C) were determined. For the latter, media were passed through 0.45-μm filters before analysis.

Fig. 6. Plasmin-mediated degradation of AgLDL during incubation with macrophages plus LPDS. Two-week-old monocyte-macrophage cultures were incubated 24 h with 50 μg/ml 125I-AgLDL in 1 ml of RPMI 1640 medium containing 10% LPDS with either 200 μg/ml purified mouse anti-plasminogen monoclonal antibody (solid bars), or 200 μg/ml purified isotype-matched control mouse monoclonal antibody (MOPC21 mouse myeloma protein) (hatched bars). Following incubations, cell-associated and medium trichloroacetic acid (TCA)-soluble 125I-AgLDL were determined.

Fig. 7. Structure of AgLDL after interaction with macrophages. 100 μg/ml AgLDL produced by phospholipase C treatment was incubated 24 h in RPMI 1640 medium containing 1 unit/ml plasminogen without (A) or with macrophages (B). Samples of culture medium were negatively stained and examined by electron microscopy. The large aggregates of LDL produced by phospholipase C treatment (A) were disaggregated during incubation with macrophages in the presence of plasminogen (B). Phospholipase C causes fusion of LDL (22) accounting for the presence of lipid particles larger than 22 nm in diameter, the usual size of LDL. Bar is 100 nm and applies to A and B.
Macrophage Reversal of LDL Aggregation

A. 2-week-old monocyte-macrophages were incubated in RPMI 1640 medium with 50 µg/ml 125I-AgLDL for 5 h to accumulate 125I-AgLDL in SCC. Then the macrophages were rinsed 3 times in RPMI 1640 medium, and incubated 1 day in RPMI 1640 with either plasminogen (1 unit/ml) or 10% LPDS. Following incubations, the trichloroacetic acid-insoluble 125I-AgLDL released into the medium was determined before and after filtration through a 0.45-µm (pore-size) polysulfone (low protein-binding) filter. This assessed the decrease in size of the original 125I-AgLDL, <10% of which could pass through the 0.45-µm filter before or after a 1-day incubation in either medium without macrophages. B. macrophages were incubated in RPMI 1640 medium with either no addition, plasminogen (1 unit/ml), or 10% LPDS for 1 day. The macrophage-conditioned medium was removed, filtered, and 50 µg of 125I-AgLDL was added to each sample of medium. After a 1-day incubation at 37 °C, the % of trichloroacetic acid-insoluble 125I-AgLDL in the medium that passed through a 0.45-µm filter was determined.

| Condition | % of trichloroacetic acid-insoluble 125I-AgLDL in medium that passed through a 0.45-µm filter |
|-----------|-------------------------------------------------------------------------------------------------|
| A. 125I-AgLDL incubated with macrophages | 68 ± 4 |
| 125I-AgLDL (vortexed) 5 h/plasminogen 1 day | 64 ± 4 |
| 125I-AgLDL (vortexed) 5 h/LPDS 1 day | 62 ± 3 |
| 125I-AgLDL (vortexed) 5 h/LPDS 1 day | 67 ± 5 |
| B. 125I-AgLDL (vortexed) incubated in medium conditioned by macrophages | 4 ± 0 |
| Macrophages + no addition 1 day/conditioned medium + 125I-AgLDL 1 day | 72 ± 8 |
| Macrophages + plasminogen 1 day/conditioned medium + 125I-AgLDL 1 day | 8 ± 0 |

Macrophage degradation of the disaggregated 125I-AgLDL occurred as shown by the finding that there was no production of trichloroacetic acid-soluble 125I-tyrosine. Plasmin treatment of LDL is reported not to affect its recognition and uptake through the LDL receptor (37). Therefore, we tested whether macrophages could degrade native 125I-LDL. Macrophages also did not degrade fresh 125I-LDL (16 µg/ml) added to macrophage-conditioned medium without aprotinin and incubated similarly for 5 h with fresh macrophages. The lack of degradation of 125I-LDL and macrophage-disaggregated 125I-AgLDL is consistent with down-regulation of the LDL receptor reported to occur in differentiated human monocyte-macrophages (38). Indeed, confirming what we reported previously (15), uptake of AgLDL into human monocyte-macrophages was not mediated by the LDL receptor. An anti-LDL receptor antibody (200 µg/ml) that blocks LDL interaction with the LDL receptor did not decrease uptake of 125I-AgLDL (50 µg/ml) incubated 5 h with macrophages.

Human fibroblasts degrade 125I-LDL taken up by the LDL receptor that is well expressed in these cells. Therefore, we compared degradation of 125I-LDL (8.5 µg/ml) and macrophage-disaggregated 125I-AgLDL (8.5 µg/ml) incubated 24 h with human fibroblasts. Human fibroblasts degraded similar amounts of 125I-LDL and macrophage-disaggregated 125I-AgLDL, 2.1 ± 0.1 and 2.0 ± 0.1 µg/ml, respectively. Degradation was inhibited by a 23-fold excess of unlabeled LDL showing that the degradation was specific and mediated by the LDL receptor.

DISCUSSION

Uptake of AgLDL into macrophages by patocytosis led to its storage in SCC until macrophages were exposed to serum. Then, disaggregation and release of accumulated AgLDL occurred following macrophage-mediated conversion of serum-derived plasminogen to active plasmin. On the other hand, if macrophages encountered AgLDL in the presence of serum, the macrophages initially accumulated AgLDL but over time released disaggregated LDL due to macrophage activation of serum plasminogen. Although it has been reported that macrophages possess both the urokinase and tissue types of plasminogen activators (29), only the urokinase-type of plasminogen activator-mediated plasminogen activation was involved in release and disaggregation of AgLDL. A monoclonal antibody that inhibits urokinase plasminogen activator effectively blocked release of AgLDL from macrophages, while a monoclonal antibody that inhibits tissue plasminogen activator did not block AgLDL release.

How does plasmin cause release of AgLDL from macrophage SCC? Previously, we showed that trypsin could release AgLDL that had accumulated in SCC (15). Both trypsin and plasmin can partially degrade apoB of LDL (36) and disaggregate AgLDL in the absence of macrophages. Here, macrophage-generated plasmin caused partial degradation of LDL protein (i.e. apoB). While some LDL contained in AgLDL may bind SCC directly, most LDL in AgLDL is presumably retained in SCC because of adherence of one LDL to another LDL within AgLDL. It is likely that plasmin degradation of apoB disrupts the non-covalent bonds that hold LDL in aggregates and causes LDL to disaggregate. Because SCC are open to the extracellular space, LDL particles disaggregated by plasmin can diffuse from SCC into the extracellular space. It is unlikely that macrophage-generated plasmin induced an active expulsion of LDL from SCC as neither cytochalasin D nor nocodazole (inhibitors of microfilaments and microtubules, respectively) blocked LDL release.

Interaction of AgLDL with macrophages was necessary for reversal of its aggregation in serum. This finding is consistent with previous reports that only plasmin bound to the macrophage surface is active, because when bound, plasmin is protected from the action of serum inhibitors (28). Because only macrophage-bound plasmin is active in the presence of serum, it makes sense that plasmin should be closely associated with SCC where AgLDL accumulates. Unfortunately, we have not found an anti-plasmin antibody suitable for carrying out electron immunocytochemistry to investigate this issue.

While macrophage-generated plasmin increased degradation of AgLDL in the presence of serum, macrophage-generated plasmin did not cause degradation of native (monomeric) LDL. Monomeric LDL does not enter macrophage SCC (15), and monomeric LDL is not substantially metabolized by well differentiated human monocyte-macrophages owing to limited expression of the LDL receptor (38). On the other hand, AgLDL uptake into SCC of human monocyte-macrophages is not mediated by the LDL receptor (15). Thus, the finding that macrophage-generated plasmin could not degrade monomeric LDL might be because monomeric LDL does not come in contact with macrophage-bound plasmin.

After macrophage accumulation of AgLDL, exposure of these macrophages to LPDS did not result in plasmin-mediated release of all accumulated cholesterol (see Table III). This could have occurred for several reasons. First, some AgLDL undergoes lysosomal degradation during and after incubation of macrophages with AgLDL (15). Cholesterol derived from

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**TABLE III**

**Macroplage reversal of LDL aggregation**

| Condition | % of trichloroacetic acid-insoluble 125I-AgLDL in medium that passed through a 0.45-µm filter |
|-----------|-------------------------------------------------------------------------------------------------|
| A. 125I-AgLDL incubated with macrophages | 68 ± 4 |
| 125I-AgLDL (vortexed) 5 h/plasminogen 1 day | 64 ± 4 |
| 125I-AgLDL (vortexed) 5 h/LPDS 1 day | 62 ± 3 |
| 125I-AgLDL (vortexed) 5 h/LPDS 1 day | 67 ± 5 |
| B. 125I-AgLDL (vortexed) incubated in medium conditioned by macrophages | 4 ± 0 |
| Macrophages + no addition 1 day/conditioned medium + 125I-AgLDL 1 day | 72 ± 8 |
| Macrophages + plasminogen 1 day/conditioned medium + 125I-AgLDL 1 day | 8 ± 0 |

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**How does plasmin cause release of AgLDL from macrophage SCC? Previously, we showed that trypsin could release AgLDL that had accumulated in SCC (15). Both trypsin and plasmin can partially degrade apoB of LDL (36) and disaggregate AgLDL in the absence of macrophages. Here, macrophage-generated plasmin caused partial degradation of LDL protein (i.e. apoB). While some LDL contained in AgLDL may bind SCC directly, most LDL in AgLDL is presumably retained in SCC because of adherence of one LDL to another LDL within AgLDL. It is likely that plasmin degradation of apoB disrupts the non-covalent bonds that hold LDL in aggregates and causes LDL to disaggregate. Because SCC are open to the extracellular space, LDL particles disaggregated by plasmin can diffuse from SCC into the extracellular space. It is unlikely that macrophage-generated plasmin induced an active expulsion of LDL from SCC as neither cytochalasin D nor nocodazole (inhibitors of microfilaments and microtubules, respectively) blocked LDL release.**

Interaction of AgLDL with macrophages was necessary for reversal of its aggregation in serum. This finding is consistent with previous reports that only plasmin bound to the macrophage surface is active, because when bound, plasmin is protected from the action of serum inhibitors (28). Because only macrophage-bound plasmin is active in the presence of serum, it makes sense that plasmin should be closely associated with SCC where AgLDL accumulates. Unfortunately, we have not found an anti-plasmin antibody suitable for carrying out electron immunocytochemistry to investigate this issue.

While macrophage-generated plasmin increased degradation of AgLDL in the presence of serum, macrophage-generated plasmin did not cause degradation of native (monomeric) LDL. Monomeric LDL does not enter macrophage SCC (15), and monomeric LDL is not substantially metabolized by well differentiated human monocyte-macrophages owing to limited expression of the LDL receptor (38). On the other hand, AgLDL uptake into SCC of human monocyte-macrophages is not mediated by the LDL receptor (15). Thus, the finding that macrophage-generated plasmin could not degrade monomeric LDL might be because monomeric LDL does not come in contact with macrophage-bound plasmin.

After macrophage accumulation of AgLDL, exposure of these macrophages to LPDS did not result in plasmin-mediated release of all accumulated cholesterol (see Table III). This could have occurred for several reasons. First, some AgLDL undergoes lysosomal degradation during and after incubation of macrophages with AgLDL (15). Cholesterol derived from
Atherosclerotic lesions, and modified or native LDL increase lipid particles that resemble LDL chemically but that are aggregated and fused LDL is one mechanism that could contribute to the accumulation of lesions in atherosclerotic lesions compared with normal, plasmin generation may be confined to areas of lesions where plasminogen activation is not inhibited by PAI-1 (32, 49–53). The state of aggregation of LDL surrounding macrophages has not yet been examined with freeze-etch microscopy, the only technique that can detect lipoprotein particle aggregation. Other types of immunohistochemical and routine microscopic studies of LDL in lesions cannot distinguish whether lipoprotein particles are aggregated or are packed together without being aggregated.

Efflux of lipoprotein particles from the vessel wall (including those as large as the fused LDL particles observed in the present study) is inversely proportional to their size (54). Therefore, aggregation of LDL in atherosclerotic lesions could contribute to LDL accumulation in lesions. Accordingly, plasmin-mediated disaggregation of LDL aggregates might facilitate efflux of LDL from the vessel wall. In this regard, plasmin has been shown to release a substantial fraction of LDL trapped in minced atherosclerotic plaque tissue samples (55). On the other hand, because macrophage emigration from lesions occurs (56), plasmin-mediated release of AgLDL from macrophages precludes the possibility of macrophages transporting AgLDL out of lesions. Further study is needed to learn the significance of macrophage-mediated AgLDL disaggregation for the development of atherosclerotic lesions. In any case, macrophage disaggregation of aggregated and fused LDL is a novel mechanism for generating size-consistent models of lipoprotein structures found in atherosclerotic lesions.

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Macrophage Reversal of LDL Aggregation

AgLDL degraded in lysosomes could remain within macrophages. Second, exposure of macrophages to serum did not release all cell-associated AgLDL from macrophages, and thus some AgLDL may remain in SCC. Last, Tabas and colleagues (39) have recently shown that when macrophages are incubated with AgLDL, LDL-cholesterol ester hydrolysis exceeds LDL protein degradation, a phenomenon associated with selective cellular uptake of cholesteryl esters from monomeric LDL in other studies (40). Possibly, some cholesterol transfers from AgLDL in SCC into the macrophage cytoplasm by this selective uptake process. In any case, it remains to be determined under what conditions AgLDL can transform human monocyte-derived macrophages into foam cells where most accumulated cholesteryl ester is stored in cytoplasmic lipid droplets. Under the conditions examined so far, most AgLDL (and its cholesterol) that enters human monocyte-macrophages by patocytosis remains in macrophage SCC, or is disaggregated and released when these macrophages are exposed to plasminogen in serum.

What is the fate of disaggregated AgLDL particles released from macrophages? While it is possible that disaggregated AgLDL released from macrophage SCC could re-enter macrophages through other endocytic pathways such as coated pits and undergo lysosomal degradation, this did not occur during incubation of macrophages with the concentrations of disaggregated AgLDL that we could test. Incubation of disaggregated 125I-AgLDL with fresh macrophages in medium lacking plasmin activity did not produce any additional degradation of the disaggregated 125I-AgLDL. However, 125I-LDL also was not degraded significantly when incubated with macrophages under similar conditions (i.e. low 125I-LDL concentration and short time of incubation that should detect LDL receptor-mediated uptake rather than low affinity and fluid-phase uptake of 125I-LDL). As mentioned above, lack of significant degradation likely reflects down-regulation of the LDL receptor in differentiated monocyte-derived macrophages (38). Macrophage-released disaggregated 125I-AgLDL and 125I-LDL were degraded similarly by human fibroblasts showing that LDL receptor-mediated uptake of disaggregated 125I-AgLDL could occur. Although macrophage-disaggregated AgLDL potentially can be metabolized through the LDL receptor, this receptor is down-regulated in cells of human atherosclerotic lesions (41). Thus, if disaggregated AgLDL occurs in atherosclerotic lesions, the lipid particles could accumulate in the extracellular spaces of lesions similar to their accumulation in culture medium here.

Aggregates of spherical particles, some having larger diameters (up to 118 nm) than LDL (22 nm), have been observed by freeze-etch analysis in rabbit subendothelial matrix following injection of monomeric LDL (5), and during atherosclerotic lesion development in Watanabe heritable hyperlipidemic rabbits that have genetically elevated LDL levels (7). Similar-sized non-aggregated cholesteryl ester-rich lipoprotein structures occur in atherosclerotic lesions, and do not appear to be derived from release of intracellular lipid droplets (7, 18, 42–45). Here, disaggregated AgLDL released by macrophages also showed particles with diameters larger than LDL. The presence of particles larger than LDL that were not aggregates of LDL can be attributed to the fact that vortexing, sphingomyelinase, and phospholipase C treatment of LDL cause LDL fusion as well as aggregation (20–22, 46). Thus, macrophage disaggregation of aggregated and fused LDL is one mechanism that could contribute to the accumulation in lesions of those extracellular lipid particles that resemble LDL chemically but that are larger than LDL.

Plasminogen derived from the blood has been detected in atherosclerotic lesions, and modified or native LDL increase the capacity of some types of macrophages to produce plasmin (47, 48). Also, we found that macrophage cholesterol accumulation did not inhibit plasminogen-dependent release and disaggregation of AgLDL. Thus, since macrophages reverse aggregation of LDL in the presence of plasminogen in vitro, one might expect disaggregated AgLDL in areas where macrophages are present. However, conversion of plasminogen to active plasmin by macrophages could be limited. PAI-1 inhibited serum plasminogen-induced release of AgLDL from macrophages. Considering that levels of PAI-1 are increased in atherosclerotic lesions compared with normal, plasmin generation may be confined to areas of lesions where plasminogen activation is not inhibited by PAI-1 (32, 49–53). The state of aggregation of LDL surrounding macrophages has not yet been examined with freeze-etch microscopy, the only technique that can detect lipoprotein particle aggregation. Other types of immunohistochemical and routine microscopic studies of LDL in lesions cannot distinguish whether lipoprotein particles are aggregated or are packed together without being aggregated.
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