Aggregated Low Density Lipoprotein Induces and Enters Surface-connected Compartments of Human Monocyte-Macrophages

UPTAKE OCCURS INDEPENDENTLY OF THE LOW DENSITY LIPOPROTEIN RECEPTOR* (Received for publication, July 21, 1997, and in revised form, September 23, 1997)

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Aggregation of low density lipoprotein (LDL) stimulates its uptake by macrophages. We have now shown by electron microscopic and chemical experiments that aggregated LDL (produced by vortexing (VxLDL) or treatment with phospholipase C) induced and became sequestered in large amounts within surface-connected compartments (SCC) of human monocyte-derived macrophages. This occurred through a process different from phagocytosis. Formation of SCC and accumulation of aggregated LDL in SCC are cell-mediated processes that were temperature-dependent (10 × greater cell association at 37 °C than at 4 °C) and blocked by cytochalasin D but not by nocodazole. Because of the surface connections of SCC, trypsin could release aggregated LDL from SCC. Degradation of 125I-VxLDL through the SCC pathway showed delayed and a lower rate of degradation compared with nonaggregated 125I-acetylated LDL that did not enter SCC. However, similar to 125I-acetylated LDL degradation, 125I-VxLDL degradation occurred through a chloroquine-sensitive pathway. Uptake of VxLDL into SCC was not mediated by the LDL receptor. Methylation of LDL prevents its binding to the LDL receptor. However, methylated LDL still entered SCC after it was aggregated by vortexing. On the other hand, degradation of 125I-VxLDL was substantially decreased by methylation of LDL and by cholesterol enrichment of macrophages, which decreases macrophage LDL receptor expression. The results suggest that whereas uptake of aggregated LDL into SCC occurs independently of the LDL receptor, movement of aggregated LDL from SCC to lysosomes may depend in part on LDL receptor function. Sequestration into SCC is a novel endocytotic pathway for uptake of aggregated LDL that allows the macrophage to store large amounts of this lipoprotein before it is further processed.

Previously, we reported that human monocyte-macrophages display an unusual mode of endocytosis in which they sequester cholesterol crystals into an extensive labyrinth of cytoplasmic compartments (1). These compartments remain connected to the surface of the macrophage and to the extracellular space. Thus, this endocytotic process is different from phagocytosis, where particles are taken into the macrophage within vacuoles that form from pinched-off regions of the plasma membrane and do not maintain any connection to the extracellular space (2).

Besides cholesterol crystals, we observed that acetylated low density lipoprotein (AcLDL)1 could induce and enter SCC of human monocyte-macrophages (1). However, we subsequently discovered that this occurred only for some lots of AcLDL and not with other lots. AcLDL that entered these compartments was aggregated in linear strands. We suspected that some aggregation of AcLDL occurred during its storage and that this aggregated AcLDL could have been responsible for the formation of the macrophage SCC.

Other investigators have shown that aggregation of lipoproteins enhances their uptake by macrophages. Oxidation and thiolation of LDL or treatment of LDL with sialidase, phospholipase C, or sphingomyelinase plus lipoprotein lipase (in the presence of cultured smooth muscle cells) and other treatments cause LDL to aggregate and to show enhanced degradation by macrophages (3–10). Khoo et al. (11) showed that aggregation of normal LDL by vortexing converted LDL to a form that was readily taken up by mouse peritoneal macrophages through an LDL receptor-dependent mechanism. The endocytotic pathway for uptake of the vortexed low density lipoprotein (VxLDL) was not described, but it was suggested that this LDL entered the macrophages by phagocytosis.

In the present study, we examined whether aggregated LDL has the capacity to induce and accumulate within monocyte-macrophage SCC. Not only did aggregated LDL induce and accumulate within SCC, this occurred by a mechanism that did not depend on the classical LDL receptor.

MATERIALS AND METHODS

Preparation of Lipoproteins—Human LDL was prepared as described previously (12) and was obtained from PerImmune (Rockville, MD). AcLDL was prepared as described by Basu et al. (13). Methylation of LDL was performed according to Weisgraber et al. (14). 125I-Labeled LDL and AcLDL with specific activities that ranged 70–200 μCi/mg of protein were obtained from Biomedical Technologies (Stoughton, MA). Lipoproteins that were not to be aggregated by vortexing were centrifuged at 14,000 × g for 10 min to remove any spontaneously formed aggregates from these lipoprotein preparations. To prepare aggregated lipoproteins, 40 μL of LDL or methylated LDL (5 mg/ml for unlaabeled and 1–3 mg/ml for labeled lipoproteins) was placed into a 0.5-ml silicon-coated polystyrene tube and vortexed (VWR vortex mixer) at the maximal speed for 1 min. LDL aggregated with phospholipase C treatment was prepared as described previously (7, 8).

Assay of Cell Association and Degradation of 125I-Lipoproteins—The cell association and degradation of lipoproteins by human monocyte-derived macrophages was performed according to the methods of Goldstein et al. (15) using 125I-VxLDL and 125I-AcLDL. Human monocyte-derived macrophages were cultured as described previously except that

1 The abbreviations used are: AcLDL, acetylated low density lipoprotein; VxLDL, vortexed LDL; SCC, surface-connected compartments; DPBS, Dulbecco’s phosphate-buffered saline; VLDL, very low density lipoprotein.
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2 × 10^6 monocytes/well were initially seeded into 12-well (22-mm diameter) culture plates (Plastek C, MatTek Corp., Ashland, MA) (12). Two-week-old monocyte-macrophage cultures were rinsed three times with RPMI 1640 medium and incubated for the indicated times at 37 °C with 125I-labeled lipoproteins added to RPMI 1640 medium containing 0.2% fatty acid-free bovine serum albumin.

Lipoprotein degradation was quantified by measurement of trichloroacetic acid-soluble organic iodide radioactivity in supernatants of media samples that were centrifuged at 15,000 × g for 10 min. Values obtained in the absence of cells were <5% those values obtained with cells. These control values were subtracted. Cell-associated 125I-lipoproteins were determined by rinsing macrophages 5 times with Dulbecco's phosphate-buffered saline (DPBS) containing Ca^{2+} and Mg^{2+}, and then 0.35% bovine serum albumin (3 quick rinses and two 10-min incubations on ice). After a final rinse with DPBS plus Ca^{2+} and Mg^{2+}, macrophages were dissolved overnight in 0.1 N NaOH. Aliquots of NaOH-solubilized cell samples were assayed for 125I radioactivity. Values of 125I radioactivity determined for wells incubated with 125I-lipoproteins but without macrophages were subtracted. These values were <1% those of the cell-associated 125I-lipoproteins. Macrophage protein content was determined by rinsing macrophages 5 times with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and then for another 2 days with Dulbecco's modified Eagle's medium containing 10% human lipoprotein-deficient serum (PerImmune) before incubations with 125I-lipoproteins were carried out.

Trypsin Release of 125I-Lipoproteins from SCC—After incubation with the indicated amount of 125I-lipoprotein, monocyte-macrophages were rinsed three times with RPMI 1640 and incubated for 30 min in 1 ml of RPMI 1640 with 25 µl of DPBS plus Ca^{2+} and Mg^{2+} or that solution containing 50 µg of trypsin (2,740 units/mg of protein; Life Technologies Inc.) at 37 °C. Then cells were rinsed and assayed for cell-associated 125I-lipoprotein and cell protein. Organic 125I-radioactivities in soluble and trichloroacetic acid-insoluble fractions of media were also measured.
membranous compartments most likely are SCC that were not open to the extracellular space at the time of fixation of the macrophages. The lipoprotein particles in all SCC were often larger than native LDL (which is about 22 nm) and often resembled beads on a string. The increased size of LDL particles can be explained by fusion of LDL particles induced by vortexing (19). LDL that aggregated by treatment with phospholipase C also induced and accumulated within SCC (Fig. 1c). The aggregates produced by phospholipase C were more rounded rather than linear as was VxLDL. No macrophage SCC were induced by LDL that had not been vortexed or by AcLDL centrifuged to remove any spontaneously formed aggregates.

Comparison of Metabolism of 125I-VxLDL, 125I-AcLDL, and 125I-LDL—Because VxLDL entered SCC and centrifuged AcLDL did not enter these compartments, it was possible to compare the metabolism of AcLDL and VxLDL as they were processed by two different endocytosis pathways. The time courses of the metabolism of 125I-VxLDL and 125I-AcLDL were compared. Cell association of 125I-VxLDL and 125I-AcLDL both began to reach a plateau by 2 h. However, the cell association of 125I-VxLDL was greater than 5-times that of 125I-AcLDL by 72 h of incubation with 50 μg/ml 125I-VxLDL (see Fig. 4 for results of one experiment). Nocodazole, an inhibitor of microtubule function, did not inhibit cell association (data not shown). Electron microscopy showed that cytochalasin D blocked formation of SCC induced by VxLDL similar to what we have observed for compartments induced by cholesterol crystals (1). The temperature dependence and cytochalasin D sensitivity of 125I-VxLDL cell association showed that most cell association occurred through an active cell-mediated process and was not due to nonspecific sticking of lipoprotein aggregates to the culture dish.

Aggregated LDL That Had Accumulated in SCC Could Be Released by Trypsin—After cell association of 20 μg/ml 125I-VxLDL with macrophages for 5 h, then up to 90% could be released from the macrophages with subsequent trypsin treatment (Fig. 5). Incubation of macrophages for 5 h with 50 μg/ml 125I-VxLDL showed an average (of three experiments) cell association of 19.9 ± 1.3 μg 125I-VxLDL/mg of cell protein, 67 ± 2% of which could be released by trypsin. There was a progressive decrease with time in the percentage of cell-associated 125I-VxLDL, that could be released by trypsin (Fig. 4). 86, 70, and 64% cell-associated 125I-VxLDL could be released after 1-, 3-, and 5 h-incubations with 50 μg/ml 125I-VxLDL, respectively. Between 5 and 24 h, there was no further decrease in the percent of cell-associated 125I-VxLDL that could be released by trypsin. Cell association of 125I-LDL that had been aggregated with phospholipase C was less than that of 125I-VxLDL. However, similar to 125I-VxLDL, most cell association of phospho-
were incubated for 5 h with 20 μg/ml aggregated LDL. This allowed for the detachment of macrophages from the culture surface because the lipase C-treated aggregated LDL was inhibited by cytochalasin D and could be released by trypsin (Fig. 6).

About one-third of cell-associated aggregated LDL did accumulate within macrophages by an actin-dependent process. This was shown by incubating macrophages for 5 h with 50 μg/ml aggregated LDL in the presence of cytochalasin D and then exposing the macrophages to trypsin. In this case, release of cell-associated aggregated LDL was almost complete, being 4 ± 0% of the amount found with macrophages not treated with cytochalasin D or trypsin. The occurrence of some trypsin-resistant cell-associated aggregated LDL could be explained by the electron microscopic findings of some LDL having accumulated within ruthenium red negative SCC that lacked connections to the cell surface (see electron microscopy results above), incomplete release by trypsin of LDL from open SCC, and some uptake of LDL into non-SCC endocytic compartments. However, at a minimum, one-third LDL was taken up into SCC (i.e. one-third of cell-associated aggregated LDL was both cytochalasin D-inhibitable and trypsin-releasable).

Degradation of Aggregated LDL within SCC—Degradation of cell-associated aggregated LDL was incomplete. When macrophages were incubated with 50 μg/ml aggregated LDL for 5 h and then incubated in RPMI 1640 medium without LDL for 1 and 2 days, only 38.2 ± 7.1% (n = 5 experiments) and 39.0 ± 9.6% (n = 3 experiments), respectively, cell-associated aggregated LDL was subsequently degraded.

Two-thirds of the degradation of cell-associated aggregated LDL could be accounted for by the degradation of the pool of aggregated LDL that was trypsin-releasable (i.e. was within SCC or associated with macrophage cell surfaces). This was shown by incubating monocyte-macrophages with 50 μg/ml aggregated LDL for 5 h to produce a large pool of cell-associated aggregated LDL. Then, the macrophages were treated without or with trypsin for 30 min as described in Fig. 4 to remove aggregated LDL from SCC. The amount of aggregated LDL degraded during a subsequent 24-h incubation in RPMI 1640 medium was determined for those macrophages that had their aggregated LDL removed by trypsin treatment. Trypsin treatment removed 66 ± 1% cell-associated aggregated LDL, and this decreased subsequent aggregated LDL degradation during the chase period by a similar amount at 64 ± 1%.

Degradation of aggregated LDL occurred in lysosomes. When macrophages were first incubated 5 h with aggregated LDL and then chased 1 day in RPMI 1640 medium with the lysosome inhibitor chloroquine, degradation of cell-associated aggregated LDL...
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TABLE I
Effect of chloroquine on degradation of 125I-VxLDL

| Condition                           | 125I-VxLDL 5 h | 125I-VxLDL 5 h/RPMI 1640 without chloroquine 1 day | 125I-VxLDL 5 h/RPMI 1640 with chloroquine 1 day |
|-------------------------------------|----------------|---------------------------------------------------|-------------------------------------------------|
|                                     | 16.6 ± 2.1     | 8.9 ± 0.2                                         | 13.3 ± 1.0                                       |

TABLE II
Role of LDL receptor in metabolism of 125I-VxLDL

| Condition                           | Cell-associated 125I-LDL | Degraded 125I-lipoprotein |
|-------------------------------------|--------------------------|---------------------------|
| Monocyte-macrophages 125I-LDL 5 h   | 0.1 ± 0.0                | 0.1 ± 0.0                 |
| methylated 125I-LDL 5 h             | 0.3 ± 0.1                | 0.0 ± 0.0                 |
| normal 125I-VxLDL 5 h               | 6.4 ± 0.1                | 1.2 ± 0.1                 |
| methylated 125I-VxLDL 5 h           | 9.8 ± 0.7                | 0.3 ± 0.0                 |
| methylated 125I-VxLDL + cytochalasin D 5 h | 2.0 ± 0.4 | 0.0 ± 0.0 |
| Fibroblasts 125I-LDL                | 0.3 ± 0.1                | 0.4 ± 0.0                 |
| methylated 125I-LDL                 | 0.1 ± 0.0                | 0.0 ± 0.0                 |
| Monocyte-macrophages AcLDL 2 day/125I-VxLDL 5 h | 9.6 ± 0.4 | 3.0 ± 0.2 |
| +AcLDL 2 day/125I-VxLDL 5 h         | 8.6 ± 0.2                | 0.4 ± 0.1                 |

DISCUSSION

We have shown that LDL aggregated by vortexing or treatment with phospholipase C (and vortexed AcLDL)2 induced and accumulated within SCC of human monocyte-derived macrophages. Uptake of aggregated LDL into SCC is a novel endocytosis pathway for this lipoprotein. Native nonaggregated LDL did not induce nor enter these compartments. Uptake into SCC was temperature- and actin-dependent. VxLDL that accumulated in SCC was degraded slowly compared with AcLDL. Though cell association of VxLDL was an active cell-mediated process, nevertheless cell-associated VxLDL could be released from macrophages by trypsin. This confirmed the electron microscopic finding that VxLDL had accumulated within SCC rather than within phagocytic vacuoles.

Accumulation of aggregated lipoproteins in SCC may have implications for the development of atherogenesis. The presence of aggregated lipoproteins in SCC could lead to the formation of foam cells, which are a precursor to the development of atherosclerotic plaques. Further studies are needed to investigate the role of aggregated lipoproteins in the development of atherosclerosis.

W.-Y. Zhang, P. M. Gaynor, and H. S. Kruth, unpublished data.
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Previously, uptake of aggregated lipoproteins has been attributed to the process of phagocytosis. This was because uptake of aggregated lipoproteins was inhibited by cytochalasins and phagocytosis is an actin-dependent process that is inhibitable by cytochalasins (20). Actin dependence does not necessarily mean that phagocytosis is the uptake pathway. This is because, besides phagocytosis, other endocytic pathways of macrophages such as sequestration into SCC and macropinocytosis are also actin-dependent (1, 24). Electron microscopy was not used in many previous studies to confirm that aggregated lipoproteins entered macrophages by direct phagocytosis. Not only did cytochalasin D inhibit uptake of VxLDL into SCC, cytochalasin D also inhibited subsequent degradation of SCC-bound VxLDL. Thus, it remains possible that VxLDL first entered SCC but then was transported from SCC into the cell by cytoplastosis or phagocytosis. However, we were not able to visualize by electron microscopy transport of VxLDL out of SCC, possibly because this process was slow and limited.

Uptake into SCC is a process different from phagocytosis. Phagocytosis involves uptake of material into vacuoles that form from pinched-off regions of the plasma membrane (2). SCC form in part through invaginations of the plasma membrane that do not pinch off from the plasma membrane (1). The formed SCC can be delineated with the electron-dense stain ruthenium red that distinguishes intra- from extracellular membranes by staining only the latter. Hoff et al. (25, 26) showed rapid degradation of aggregates of 4-hydroxynonenal-modified LDL taken up by mouse peritoneal macrophages through phagocytosis (verified by electron microscopy). On the other hand, these investigators reported that VxLDL showed delayed processing (i.e. degradation) by macrophages (Ref. 27; also shown in Ref. 23). The delayed processing of VxLDL was not due to a diminished capacity of lysosomal enzymes to degrade VxLDL (also shown in Ref. 28). They speculated that poor processing of VxLDL could have been due to the nature of the intracellular pathway taken by the VxLDL. Uptake of VxLDL into SCC is an intracellular pathway showing slow degradation that could explain these earlier findings.

Human monocyte-macrophages are capable of directly phagocytosing 0.5–3-μm latex beads. So what property of VxLDL targets this aggregate to SCC rather than to phagocytic vacuoles? VxLDL is multivalent with respect to its apolipoprotein B component, and valency of a ligand may target receptors to different endocytosis pathways. Tabas and co-workers (29, 30) described a unique endocytotic pathway for β very low density lipoproteins (VLDL) in mouse peritoneal macrophages in which multi-valency of ligand was a factor determining the route of endocytosis. In this pathway, large β-VLDL enter mouse peritoneal macrophages through surface-connected tubules. Surface-connected tubules are plasma membrane invaginations that do not terminate in a labyrinth of interconnected compartments as do SCC. In contrast to the uptake of VxLDL into SCC in the present study, uptake of β-VLDL into surface-connected tubules is not inhibited by cytochalasin D. Also, uptake into these tubules occurs through the LDL receptor that binds the multivalent apolipoprotein E component of β-VLDL (30). Thus, uptake of β-VLDL by mouse peritoneal macrophages through the surface-connected tubule pathway and uptake of VxLDL by human monocyte-macrophages through the SCC pathway appear to be different but possibly related endocytic pathways.

It is possible that the specific macrophage uptake pathway taken by particles depends on the nature of the receptor that binds the particles. The receptor that mediated cell association of VxLDL was not the classical LDL receptor. Methylation of LDL, which prevents binding of LDL to the LDL receptor (21), did not block macrophage cell association of methylated 125I-VxLDL, cholesterol accumulation by macrophages incubated with methylated VxLDL, or accumulation of methylated VxLDL within SCC. Similar to what Khoo et al. (11) found with mouse peritoneal macrophages, we observed that the LDL receptor did mediate much of the degradation of 125I-VxLDL. Macrophage degradation of 125I-VxLDL was partially inhibited by methylation of LDL and by macrophage cholesterol enrichment, a treatment previously shown to down-regulate the LDL receptor in macrophages (22). The results suggest that whereas uptake into SCC occurs independently of the LDL receptor, subsequent movement from SCC to lysosomes may depend in part on LDL receptor function.

It is conceivable that vortexing of LDL reveals a binding domain in LDL that is normally masked. Such a domain could then mediate LDL binding to some other receptor capable of uptake into SCC. In this regard, Gianturco et al. (31, 32) have shown the existence of a macrophage receptor that recognizes a binding domain within apolipoprotein B-48 but which is not expressed by native LDL. Alternatively, vortexing of LDL could reveal a cryptic lipid ligand. In this regard, uptake of oxidized LDL appears to be mediated in part by a lipid moiety of the oxidized LDL (33). Trypsin treatment of the VxLDL in our study did prevent its uptake by monocyte-macrophages. However, trypsin treatment also reversed the aggregation of VxLDL. Thus, it was not clear whether loss of a protein ligand or loss of multi-valency of aggregated VxLDL was responsible for the lack of macrophage uptake of the trypsin-treated VxLDL.

Uptake into SCC is a pathway that leads to cellular accumulation of at least two different types of aggregated LDL (e.g. vortexed and phospholipase C-treated LDL). In preliminary experiments, we have observed macrophage uptake into SCC for some lots of oxidized LDL, another form of LDL that can aggregate (27). Considering the role of SCC in the uptake of aggregated lipoproteins will be important in future studies. Furthermore, uptake of aggregated LDL by the non-LDL receptor pathway shown in this study could be important in cellular lipoprotein uptake in atherosclerotic lesions where activity of the classical LDL receptor is down-regulated (34).

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