The Uptake and Degradation of Matrix-bound Lipoproteins by Macrophages Require an Intact Actin Cytoskeleton, Rho Family GTPases, and Myosin ATPase Activity*

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A key cellular event in atherogenesis is the interaction of macrophages with lipoproteins in the subendothelium. In vivo, these lipoproteins are bound to matrix and often aggregated, yet most cell-culture experiments explore these events using soluble monomeric lipoproteins. We hypothesized that the internalization and degradation of matrix-retained and aggregated low density lipoprotein (LDL) by macrophages may involve the actin-myosin cytoskeleton in a manner that distinguishes this process from the endocytosis of soluble LDL. To explore these ideas, we plated macrophages on sphingomyelinase-aggregated LDL bound to smooth muscle cell-derived matrix in the presence of lipoprotein lipase. The macrophages internalized and degraded the LDL, which was mediated partially by the LDL receptor-related protein. Cytochalasin D and latrunculin A, which block actin polymerization, markedly inhibited the uptake and degradation of matrix-retained LDL but not soluble LDL. Inhibition of Rho family GTPases by Clostridium difficile toxin B blocked the degradation of matrix-retained and aggregated LDL by >90% with any inhibition of soluble LDL degradation. However, specific inhibition of Rho had no effect, suggesting the importance of Rac1 and Cdc42. Degradation of matrix-retained, but not soluble, LDL was also blocked by inhibitors of tyrosine kinase, phosphatidylinositol 3-kinase, and myosin ATPase. These findings define fundamental cytoskeletal pathways that may be involved in macrophage foam cell formation in vivo but have been missed by the use of previous cell culture models.

Accumulation of macrophages in the intima is one of the key cellular events during atherogenesis (1). These cells originate from blood-borne monocytes that enter focal areas of the subendothelium, followed by differentiation into macrophages and accumulation of cholesterol (“foam cells”) (1). Specific consequences of macrophage foam cell formation include physical effects, such as intimal thickening, and biological effects, such as internalization of lipoproteins and secretion of biologically active molecules (1, 2). Indeed, many studies, including recent in vivo investigations, have provided evidence that macrophage foam cells play roles both in early atherogenesis and in late lesional events (2–5).

The accumulation of massive amounts of intracellular cholesterol ester (CE) is the hallmark of macrophage foam cell formation (1), and CE accumulation likely triggers or amplifies some of the physical and biological effects of macrophages during atherogenesis (6, 7). Our current understanding of the cellular processes involved in cholesterol loading of macrophages can be summarized as follows (8): differentiated macrophages in the subendothelium engage and internalize “atherogenic” lipoproteins, leading to lysosomal hydrolysis of lipoprotein-CE to free cholesterol (FC). Lysosomal FC rapidly distributes to cellular membranes, predominantly the plasma membrane. After the cellular FC content reaches a “threshold” level, there is transfer of the FC to the esterifying enzyme, acyl-CoA:cholesterol acyltransferase (ACAT)(9, 10), leading to the accumulation of intracellular CE.

As is evident from the above summary, the critical initiating step in macrophage foam cell formation is the engagement and internalization of subendothelial lipoproteins. How does this occur? The usual in vitro models of macrophage-lipoprotein interactions, while useful in several aspects, fail to account for some key cellular events that likely occur in vivo. In particular, most previous studies have studied foam cell formation by incubating soluble monomeric lipoproteins with monolayers of macrophages plated on tissue culture plastic. A substantial quantity of lipoproteins in lesions, however, are avidly bound to matrix (11–13). Furthermore, both biochemical and morphological studies of human and animal lesions have shown that many of the matrix-bound lipoproteins are in a fused or aggregated state (14–16). In this regard, our laboratory has provided evidence that an arterial-wall secretory sphingomyelinase (SMase) contributes to the process of subendothelial lipoprotein aggregation (17). This point is crucial, because cell culture studies have shown that aggregated/fused lipoproteins are

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** The abbreviations used are: CE, cholesteryl ester; ACAT, acyl-CoA:cholesterol acyltransferase; EBM, 2,3-butanediol monoxime; DMEM, Dulbecco’s modified Eagle’s medium; FC, free cholesterol; LDL, low density lipoprotein; LpL, lipoprotein lipase; LRP, low density receptor-related protein; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; RAP, receptor-associated protein; SMase, sphingomyelinase; BS, bovine serum albumin; ML-7, 1-(5-isouquinolinesulfonfyl)-2-methylpiperazine; LY 294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; FBS, fetal bovine serum; PI3K, phosphatidylinositol 3-kinase; OxLDL, oxidized LDL.
among the most potent inducers of massive CE loading of macrophages (16, 18–20).

Thus, macrophages in lesions most likely engage matrix-bound and often aggregated lipoproteins. In this regard there are probably unique cellular processes that occur in vivo during foam cell formation that would clearly be missed in the typical experimental model, which emphasizes receptor-mediated endocytosis. Indeed, using an experimental system in which macrophages are plated on top of matrix-retained and SMase-aggregated LDL (21), we have shown previously that there is an initial period of prolonged contact between macrophages and matrix-retained lipoproteins during which LDL-CE hydrolysis exceeds LDL-protein degradation (22). This process clearly does not occur during typical receptor-mediated endocytosis, which involves rapid uptake of whole lipoprotein particles and parallel degradation of the CE and protein moieties of the lipoproteins (22, 23).

In the current study, we have investigated the cellular events that occur after the initial contact of macrophages with retained and aggregated lipoproteins. In this second phase, which is perhaps the most important because of the quantity of internalized cholesterol, the macrophages take up large pieces of the matrix-retained and aggregated lipoproteins. We reasoned that one or more steps in this phase may involve cytoskeleton-mediated processes that are necessary to engage large lipoprotein aggregates and release them from the matrix. Such processes, which might include cellular motility, filopodia extension and retraction, and phagocytosis, would clearly distinguish this event from receptor-mediated endocytosis of soluble lipoproteins. Herein we demonstrate that the internalization of matrix-retained LDL requires actin polymerization, myosin ATPase activity, Rho family GTPases, and other signaling events that are not needed for the internalization of soluble lipoproteins. These findings define fundamental cellular processes that may be involved in foam cell formation in vivo but have been missed by the use of previous cell culture models.

**EXPERIMENTAL PROCEDURES**

**Materials**—The J774A.1 mouse macrophage cell line and bovine aortic smooth muscle cells (SMCs) were purchased from American Type Culture Collection (Manassas, VA) and Cell Applications, Inc. (San Diego, CA), respectively. Receptor-associated protein (RAP) and blocking and non-blocking antibodies against LDL receptor-related protein (LRP) were a gift from Dr. Dudley Strickland (American Red Cross, Rockville, MD). Rat anti-murine type A scavenger receptor antibody 2F8 was purchased from Serotec, Inc. (Raleigh, NC), and mouse anti-murine CD36 antibody was from Cascade Biosciences (Winchester, MA). LDL (density, 1.020–1.063 g/ml) was isolated from fresh human plasma by preparative ultracentrifugation as described previously (24). a2-Macroglobulin was purified, converted to the receptor-binding form (25) and iodinated as previously described (26, 27). ML-7 (1-[5-isoxazolyl-2-furyl]-2-methyl piperazine) was purchased from Alexis Biochemicals (San Diego, CA). Latrunculin A, genistein, and LY 294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) were from Biomol (Plymouth Meeting, PA). Lipoprotein lipase (LpL), isolated from bovine milk, was a gift from Dr. Kevin J. Williams (Thomas Jefferson University, Philadelphia, PA). Bacillus cereus sphingomyelinase (SMase), cytochalasin D, 2,3-butanedione monoxime (BDM), wortmannin, lactoferrin from bovine colostrum, bovine serum albumin (BSA, essentially fatty acid free), and Hepes were products of Sigma Chemical Co. (St. Louis, MO). LDL (density, 1.020–1.063 g/ml) was isolated from fresh human plasma by preparative ultracentrifugation as described previously (24). a2-Macroglobulin was purified, converted to the receptor-binding form (25) and iodinated as previously described (26, 27). ML-7 (1-[5-isoxazolyl-2-furyl]-2-methyl piperazine) was purchased from Alexis Biochemicals (San Diego, CA). Latrunculin A, genistein, and LY 294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) were from Biomol (Plymouth Meeting, PA). Lipoprotein lipase (LpL), isolated from bovine milk, was a gift from Dr. Kevin J. Williams (Thomas Jefferson University, Philadelphia, PA). Bacillus cereus sphingomyelinase (SMase), cytochalasin D, 2,3-butanedione monoxime (BDM), wortmannin, lactoferrin from bovine colostrum, bovine serum albumin (BSA, essentially fatty acid free), and Hepes were products of Sigma Chemical Co. (St. Louis, MO). Alexa-488-labeled phalloidin and Alexa-546 were from Molecular Probes (Eugene, OR). Carrier-free Na125I (17.4 Ci/mg) and [1-14C]oleate were from Life Technologies, Inc. (Grand Island, NY), and tissue culture flasks and plates were from Corning Glass Works (Corning, NY). Organic solvents were purchased from Fisher Scientific (Pittsburgh, PA).

**Cell Culture**—A stock culture of J774 macrophages was grown in suspension in a spinner flask in DMEM (4.5 g/liter glucose and no sodium pyruvate), supplemented with 10% heat-inactivated FBS, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

A stock culture of bovine aortic SMCs was grown in monolayer culture in the same medium as above, except with sodium pyruvate, and used for experiments. Both cell types were grown at 37 °C in a humidified atmosphere containing 5% CO2. Mouse peritoneal macrophages were harvested from the peritoneum of mice 3 days after the intraperitoneal injection of 40 μg of concanavalin A (28) and then used immediately for the experiments described below. Experiments were performed in DMEM containing 0.2% BSA except where noted.

**Preparation of Lipid-free SMC-derived Matrix**—SMCs were plated at 50,000 cells per 11-mm dish (48-well plate) and incubated for a total of 4 days (2 days post-confluent) in DMEM, 10% FBS. After three washes with DMEM, 0.2% BSA, the SMC monolayer was air-dried for 15 min and then extracted twice with 3.2% hexane-isopropanol (v/v) for 30 min. The lipid extracts were removed and discarded, and the wells were dried for 15 min at room temperature under a tissue culture hood. After washing three times with binding buffer (90 mM NaCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM Hepes, pH 7.4), the matrix was incubated with binding buffer for 1 h at room temperature to block nonspecific sites.

**Incubation of Macrophages with Matrix-retained or Non-retained 125I-LDL**—J774 or mouse peritoneal macrophages were preincubated in DMEM, 0.2% BSA in the presence or absence of LpL as indicated in figure legends; for preincubation periods longer than 30 min, the medium was buffered with 20 mM Hepes. The macrophages were then added to the top of the matrix with retained 125I-LDL at a density of 500,000 cells in 0.8 ml per 11-mm well (48-well plate), which led to a confluence of ~90–100%. In certain experiments, macrophages were preincubated as above but were first added to the matrix without LDL for 30 min, followed by the addition of 5–25 μg/ml monomeric 125I-LDL to the well. Unless noted otherwise, all incubations were performed in DMEM, 0.2% BSA for 5 h. At the end of the incubation period, the medium was collected and assayed for 125I-LDL degradation as described (30). As a control for cell-independent lipoprotein degradation, a set of triplicate wells had matrix-retained aggregated 125I-LDL or non-retained monomeric 125I-LDL, but no macrophages; these no-cell degradation values were always <10% of the degradation observed in the presence of macrophages. For the knockout macrophage and inhibitor studies, the data are expressed as a percentage of LDL degradation by wild-type or untreated macrophages, which was in the range of ~40–200 ng/well for retained and aggregated 125I-LDL and ~25–125 ng/well for non-retained soluble 125I-LDL. To quantify the amount of matrix-bound 125I-LDL, the matrix was washed three times with 0.5 ml/well ice-cold PBS containing 0.1% BSA and then once with 1 ml/well PBS, followed by solubilization in 0.5 ml/well 0.5 N NaOH at room temperature for 18–24 h. The 125I label amounts (cpm) of the solubilized material were then determined. To determine cholesteryl esterification, 5 μl of [1-14C]oleate-BSA complex (10 cpm/pmol) (30) was added to each well 30 min after the addition of...
RESULTS

Small Degradation is shown.

Other Degradation Assays—The degradation of 125I-acetyl-LDL and activated 125I-α, macroglobulin was assayed exactly as described for 125I-LDL (30).

Fluorescence Microscopy Studies—SMCs were plated on poly-L-lysine-coated coverslip dishes (~50,000 cells per 11-mm coverslip area) for a total of 4 days (2 days post-confluent) in DMEM, 10% FBS. The SMCs were rinsed three times in DMEM, 0.2% BSA and fixed in −20 °C methanol for 10 min to prevent phalloidin staining of the SMC F-actin cytoskeleton. SMC-derived matrix was then prepared as described above and, after addition of LpL, incubated with SMase-treated Alexa-546-labeled LDL. J774 macrophages were resuspended in DMEM, 0.2% BSA and then preincubated at 37 °C with either 100 μg/ml C. difficile toxin B for 6 h or 2.5 μg cytochalasin D for 20 min. The cells were then plated on the matrices containing aggregated and retained SMase-treated Alexa-546-LDL for 30 min, 1.5 h, or 5 h in the continued presence of either toxin B or cytochalasin D. After each incubation time, the wells were briefly rinsed with DMEM, 0.2% BSA and simultaneously fixed, permeabilized, and stained for F-actin using 6.6% paraformaldehyde, 0.05% glutaraldehyde, 250 μg/ml saponin, and 5 units/ml Alexa-488-labeled phalloidin in PBS for 5 min. Addition of Alexa-labeled phalloidin to the fixation/permeabilization buffer was required to stabilize as well as stain the F-actin. Fixation was then quenched by a 15-min incubation with 0.1 μg/ml glycine in PBS, followed by a final rinse in PBS. The cells were viewed with an LSM 510 laser scanning confocal microscope (Zeiss). Excitation on the LSM 510 unit was with a 25-milliwatt (mW) argon laser emitting at 488 nm and a 1.0-mW helium/neon laser emitting at 543 nm; emissions were collected using a 505- to 530-nm band pass filter to collect Alexa-488 emissions and a 585-nm long pass filter to collect Alexa-546 emissions. Typically, 0.5-μm vertical steps were used, with a vertical optical resolution of <1.0 μm.

In Vitro ADP Ribosylation Assay—Macrophages were incubated for 3 h in the presence or absence of 400 ng/ml C2IN-C3 fusion protein, C2Ha, or both components together. Next, lysates from these macrophages were incubated in vitro with [32P]NAD in the presence of C3 exoenzyme and then subjected to electrophoresis and autoradiography as described (31). In this assay, the radiographic signal is high if RhoA was unmodified at the time of cell lysis, and the signal is low if RhoA had already been ADP-ribosylated in vivo (i.e. prior to cell lysis; see “Results” section).

Statistics—Results for all bar graph values are given as means ± S.E. (n = 3); absent error bars in the bar graphs signify S.E. values smaller than the graphic symbols.

RESULTS

Initial Characterization of the Interaction of Macrophages with Matrix-retained and SMase-aggregated LDL—In an attempt to model the interaction of arterial-wall macrophages with subendothelial lipoproteins, SMase-treated 125I-LDL was first incubated with matrix derived from aortic smooth muscle cells that had been previously preincubated with lipoprotein lipase (LpL). A secretory form of SMase is present in the arterial wall, and there is evidence that this enzyme causes the aggregation and fusion of LDL that is known to occur in the subendothelium (17). LpL is also present in the subendothelium and is thought to non-enzymatically bridge LDL to matrix (32). After 18–24 h of incubation, non-bound 125I-LDL was removed, and macrophages were then added to the dish. As shown in Fig. 1A, the amount of 125I-LDL bound to the matrix was directly proportional to the concentration of 125I-LDL initially added. Typically, ~5% of the 125I-LDL added was associated with the matrix after 18–24 h, and >95% of this matrix-bound material remained bound during subsequent incubations (not shown).

When macrophages were added to different amounts of matrix-bound 125I-LDL, ~30–40% of the bound lipoprotein was degraded up to a certain point, and then no further degradation occurred (Fig. 1B). Degradation increased with time of incubation up to ~12 h (Fig. 1C). As shown in Fig. 1D, the relationship between matrix-bound LDL and stimulation of ACAT-mediated cholesterol esterification was very similar to the relationship between matrix-bound LDL and 125I-LDL degradation (compare with Fig. 1B). The similarity of these two curves is consistent with the idea that lipoprotein degradation, by increasing cellular cholesterol stores, directly drives cholesterol esterification (8).

In an additional experiment, we tested the effect of omitting LpL from the experimental system. Surprisingly, both the amount of SMase-aggregated 125I-LDL bound to the matrix and the amount of 125I-LDL degradation by macrophages was the same in the presence or absence of LpL. In contrast, omission of SMase treatment of 125I-LDL (i.e. monomeric 125I-LDL—see below) led to a 65% decrease in 125I-LDL bound to matrix and an 80% decrease in 125I-LDL degradation by macrophages. Thus, in this particular model, as opposed to one in which...
ammonium hydroxide-insoluble smooth muscle cell-derived matrix was used (21), the retention and macrophage uptake of SMase-aggregated LDL was independent of LpL.2 Treatment of LDL with SMase, however, was important for both matrix retention and degradation by macrophages.

Partial Role of LRP in the Interaction of Macrophages with Matrix-retained and SMase-aggregated LDL—The complex nature of macrophages interacting with matrix-bound aggregated lipoproteins likely involves multiple cell-surface molecules. To assess if known lipoprotein receptors play partial roles in this interaction, we conducted a series of experiments using peritoneal macrophages from receptor knockout mice or inhibitors of lipoprotein receptors. As shown in Fig. 2A, neither the LDL receptor nor CD36, a receptor for oxidized LDL (33), was necessary for the uptake and degradation of retained and aggregated LDL by mouse peritoneal macrophages. Similarly, antibodies against either CD36 or the type A scavenger receptor on J774 macrophages did not inhibit the uptake and degradation of retained and aggregated LDL by these cells (Fig. 2B); as a positive control for the anti-type A scavenger receptor antibody, we showed that it was able to block the degradation of activated 125I-acetyl-LDL by 60%. C, J774 macrophages were preincubated in the absence or presence of 100 μg/ml lactoferrin, 1 μM RAP, 86 μg/ml non-blocking anti-LRP IgG, or 86 μg/ml blocking anti-LRP IgG for 30–45 min and then incubated in the absence or presence of the same compounds with matrix-retained and SMase-aggregated 125I-LDL. After 5 h, degradation of 125I-LDL was measured. In D, a similar experiment was conducted except the macrophages were incubated with 10 μg/ml non-retained monomeric 125I-LDL (cross-hatched bar), 5 μg/ml non-retained aggregated 125I-LDL (gray bar), or 1 nM activated 125I-α2-macroglobulin (257-α2-M*, diagonal-hatched bar) in the absence (Con) or presence of 1 μM RAP (D). Degradation was assayed after 5 h for monomeric and aggregated 125I-LDL and after 3 h for 125I-α2-macroglobulin.

2 For the rest of the experiments in this study, LpL was included in the model system because it is known to be associated with matrix in vivo (32). Future studies will be needed to determine if LpL has any effects in the current model other than those involved in LDL retention and degradation.
Discussion

Lipoprotein receptors, must also play important roles (see Discussion). At the 5-h time point, clusters of aggregated LDL appeared to be internalized by the cells (Fig. 3E). In contrast, the cytochalasin D-treated macrophages (B, D, and F), although remaining attached to the matrix, were rounded and devoid of cell-surface extensions. Rather, the F-actin appeared to accumulate as a dense mass near one area of the cell surface. There was some contact with the LDL, but little evidence of internalization, even at the 5-h time point.

To assess the effect of cytochalasin D using a quantitative biochemical assay, macrophages incubated with increasing concentrations of the drug were assessed for their ability to degrade retained and aggregated LDL, as well as soluble monomeric LDL, after 5 h of incubation. As shown in Fig. 4A, cytochalasin D treatment had a dramatic, dose-dependent inhibitory effect on the uptake and degradation of retained and aggregated LDL, but there was a much smaller effect on the degradation of soluble monomeric LDL. To further investigate this point, a similar experiment was conducted using latrunculin A, which inhibits actin polymerization by a different mechanism, namely through binding actin monomers (41). Interestingly, 1 μM latrunculin A actually stimulated the uptake of non-retained monomeric LDL (cf. Refs. 42, 43), but its effect on the uptake of matrix-retained and aggregated LDL was, like cytochalasin D, strongly inhibitory (Fig. 4B). Thus, the internalization and degradation of matrix-retained and aggregated LDL by macrophages requires barbed end actin filament growth.

To determine whether matrix retention, aggregation, or both were important in the cytochalasin D effect, untreated or cytochalasin D-treated macrophages were incubated with monomeric or aggregated LDL, either in the non-retained or matrix-bound state (Fig. 4C). In the absence of matrix retention (first two bars in 4C), cytochalasin D inhibited the degradation of aggregated LDL somewhat greater than the degradation of monomeric LDL. The highest level of inhibition, however, was seen with matrix-retained LDL, whether aggregated or not (last two bars in 4C). This important finding emphasizes the importance of matrix retention and indicates that non-retained lipoprotein aggregation alone cannot explain the requirement for barbed end actin polymerization (see "Discussion").

The Importance of the Actin Cytoskeleton in the Degradation of Matrix-retained LDL—We reasoned that certain cytoskeletal processes, such as force generation, cellular motility, and/or filopodia extension, may play important roles when macrophages engage, internalize, and degrade aggregated LDL that is avidly bound to matrix. If true, this feature might distinguish the uptake of retained and aggregated lipoproteins from the endocytosis of soluble lipoproteins. Thus, we undertook a series of morphological and biochemical experiments to assess the roles of actin and myosin in the uptake and degradation of matrix-retained and SMase-aggregated LDL.

Degradation of Matrix-retained LDL by Macrophages

In the first experiment, macrophages were treated with various doses of cytochalasin D to block barbed end filament growth (40) and then incubated for up to 5 h with either retained and aggregated LDL or with monomeric non-retained LDL. In the fluorescence microscopy experiment shown in Fig. 3, macrophages were plated on top of SMase-treated, Alexa-546-labeled LDL bound to matrix. After various times of incubation, the control or cytochalasin D-treated macrophages were fixed, permeabilized, and incubated with Alexa 488-labeled phalloidin to stain actin filaments, and the cells were then visualized by confocal fluorescence microscopy. The control macrophages (A, C, and E) had prominent, F-actin-positive cell surface extensions that were in contact with retained and aggregated LDL. Note that the apparent sizes of many of the matrix-bound LDL aggregates were ≥1 μm, whereas unbound aggregates tend to be in the 100-nm range (20) (see "Discussion"). The 5-h time point, clusters of aggregated LDL appeared to be internalized by the cells (Fig. 3E). In contrast, the cytochalasin D-treated macrophages (B, D, and F), although remaining attached to the matrix, were rounded and devoid of cell-surface extensions. Rather, the F-actin appeared to accumulate as a dense mass near one area of the cell surface. There was some contact with the LDL, but little evidence of internalization, even at the 5-h time point.

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The Involvement of Rho Family GTPases in the Degradation of Matrix-retained LDL—To explore actin signaling pathways involved in the uptake and degradation of retained and aggregated LDL, we investigated the effects of inhibitors of the Rho family of GTPases. Confocal fluorescence microscopy experiments revealed that treatment of macrophages with C. difficile toxin B, which specifically monoglucosylates and inactivates Rho, Cdc42, and Rac (26), had similar effects as cytochalasin D (Fig. 5). In particular, toxin B inhibited F-actin-rich cell surface extensions (compare treated cells in right panels with control.
The concentration of 25 non-retained lipoproteins were added at a concentration of 25 μg/ml.

aggregated (aggreg), matrix-retained, or non-retained LDL as indicated; the non-retained lipoproteins were added at a concentration of 25 μg/ml.

F versus E

parent LDL internalization (Fig. 5). The results of the quantitative biochemical assay were consistent with these findings: toxin B treatment had no inhibitory effect on the degradation of monomeric non-retained LDL (cf. Ref. 44) but, remarkably, inhibited the degradation of retained and aggregated LDL by ~90% (Fig. 6A). These data further distinguish the uptake and degradation of matrix-retained lipoproteins from the endocytosis of non-retained lipoproteins.

To further examine the role of Rho family GTPases, macrophages were treated with C. sordellii lethal toxin, which monoglucosylates and inhibits Rac and possibly Cdc42, but not Rho; Ras proteins, including Ras, Rap, and Rap2, are also glucosylated by this compound (26). The degradation of retained and aggregated LDL by macrophages treated with this compound was inhibited markedly, although in this case there was also partial inhibition of the degradation of non-retained LDL (Fig. 6B), perhaps due to the effects of inhibition of Ras family proteins on receptor-mediated endocytosis (45, 46).

Lastly, to focus specifically on Rho, macrophages were exposed to C. limosum C3-like exoenzyme, which results in selective ADP ribosylation and inhibition of RhoA, RhoB, and RhoC (27). Given that C3 exotoxin itself is cell-impermeable, the strategy of Barth et al. (27) was used to facilitate internalization of C3-like ADP-ribosylation activity. Specifically, the macrophages were preincubated for 3 h and then treated for 5 h with C. botulinum CIIa together with C2IN-C3 fusion protein, which results in CIIa-mediated internalization of the enzymatically active fusion protein (27). Strikingly, the combined components had no inhibitory effect at all on the degradation of retained and aggregated LDL (Fig. 6C). To prove that the exotoxin was active, cell homogenates were assayed for ADP ribosylation of Rho using an in vitro radioisotopic assay (31). In this assay, cell lysates are incubated in vitro with [32P]NAD in the presence of C3 exoenzyme, and then subjected to electrophoresis and autoradiography. If there is abundant ADP-ribosylation in vivo (i.e. prior to cell homogenization), few sites on Rho are available for [32P]ADP ribosylation in vitro, and the Rho autoradiographic signal is low. In contrast, the signal is high in cells in which Rho was unmodified at the time of cell homogenization. As shown in Fig. 6D, Rho in cell lysates derived from macrophages incubated with no drug or with the inactive individual components of compound drug showed robust in vitro labeling, indicating that Rho was unmodified at the time of homogenization. In contrast, Rho from cells incubated with both CIIs and C2IN-C3 had a very low signal, indicating abundant ADP ribosylation in vivo. Thus, inhibition of Rho by ADP ribosylation in macrophages had no effect on the uptake and degradation of matrix-retained and aggregated LDL.

The Importance of Tyrosine Kinase, PI3K, and Myosin ATPase Activities in the Degradation of Matrix-retained LDL—Tyrosine kinases and phosphatidylinositol 3-kinases (PI3Ks)
FIG. 6. Effect of clostridial toxins on the uptake and degradation of matrix-retained and non-retained LDL by macrophages. J774 macrophages were preincubated in the absence or presence of 91 ng/ml C. difficile toxin B (A), 100 ng/ml C. sordellii lethal toxin (B), or 400 ng/ml C2IN-C3 fusion protein and C2IIa (C), which were derived from C. botulinum toxin B and C. limosum C3-like exoenzyme. The preincubation times were 6 h for toxin B and 3 h for lethal toxin and C2IN-C3/C2IIa. The macrophages were then incubated in the absence or presence of the same compounds with matrix-retained and SMase-aggregated 125I-LDL or with 5 μg/ml monomeric non-retained 125I-LDL. After 5 h, degradation of 125I-LDL was measured. D, autoradiogram of an SDS-polyacrylamide electrophoresis gel in which lysates of macrophages treated with no drugs, CIIa alone, C2IN-C3 alone, and both components together were subjected to an in-vitro ADP ribosylation assay. As explained in the text, the presence of the Rho signal in the first three lanes indicates no or little ADP ribosylation at the time of cell lysis, whereas the absence of the Rho signal in the fourth lane indicates abundant ADP ribosylation of Rho in vivo. The upper band in the first three lanes is [32P]ADP-ribosylated Rho, and the lower bands are [32P]ADP-ribosylated Rho degradation products that are sometimes seen in this assay.

represent two class of enzymes that are involved in certain actin signaling pathways mediated by the Rho family of GTPases (47, 48). In particular, the broad tyrosine kinase inhibitor genistein and the PI3K inhibitors wortmannin and LY 294002 have been shown to block specific Rho-, Rac-, and Cdc42-induced actin signaling events (47, 49–52). Thus, we determined whether these inhibitors could also differentially affect the uptake and degradation of retained and aggregated LDL versus non-retained LDL. Macrophages were preincubated for 30 min with either genistein, wortmannin, or LY 294002 and then, in the continued presence of the compounds, tested for their ability to degrade retained or non-retained LDL over a 5-h period. Although macrophages treated with these compounds became rounded, the cells remained attached to the matrix. As shown in Fig. 7A, genistein had no substantial effect on the uptake and degradation of monomeric non-retained LDL, but the degradation of aggregated retained LDL was inhibited by ~75%. With wortmannin, there was no inhibition of degradation of monomeric non-retained LDL, but the degradation of retained and aggregated LDL was inhibited by ~70% (Fig. 7B, solid bars). Treatment of the macrophages with LY 294002 had some inhibitory effect (~30%) on the degradation of monomeric non-retained LDL, but the compound inhibited the degradation of retained and aggregated LDL by ~85% (Fig. 7B, cross-hatched bars). These data further point out fundamental differences between the uptake and degradation of matrix-retained and aggregated LDL versus the endocytosis of monomeric non-retained LDL by macrophages and, pending further investigation, may indicate important roles for tyrosine kinases and PI3Ks in the former process.

Finally, to examine the potential role of myosin in the uptake and degradation of retained and aggregated LDL, macrophages were incubated in the absence or presence of BDM, an inhibitor of myosin ATPase (53), and ML-7, an inhibitor of myosin light chain kinase (54). BDM is an inhibitor of most, if not all, myosins, whereas ML-7 specifically inhibits the activity of myosin II (55). As shown in Fig. 8A, macrophages treated with BDM were blocked ~95% in their ability to degrade retained and aggregated LDL, whereas the degradation of monomeric non-retained LDL was inhibited by only ~55%. In contrast, ML-7 showed no selective inhibition: The degradation of both monomeric non-retained and aggregated retained LDL was inhibited by ~40% (Fig. 8D). These data are consistent with the idea that myosin II plays a relatively modest role in both processes but that one or more other myosins may be particularly important in the interaction of macrophages with matrix-retained and aggregated LDL.

DISCUSSION

This report is the third in a series of studies designed to investigate the interaction of arterial-wall macrophages with atherogenic lipoproteins bound to matrix, which is the state of the majority of lipoproteins in atherosclerotic lesions (11–13). In the first study, we showed that macrophages plated on top of SMase-aggregated and matrix-retained LDL internalized and degraded this material over a 24-h period and accumulated large amounts of intracellular, ACAT-derived CE (21). In the second study, we focused on the very earliest events that occur when macrophages first engage matrix-retained lipoproteins (22). During this period, there is prolonged cell-surface contact...
The internalization of retained and aggregated lipoproteins, including requirement for barbed end actin filament growth (56), dependence on Rac and Cdc42 but not Rho in the case of Fc receptor-mediated phagocytosis (57), the involvement of myosins in addition to or other than myosin II (58, 59), and sensitivity to inhibitors of tyrosine and PI3K activities (56). Although the size of non-retained SMase-aggregated LDL (~100 nm) would be too small to elicit a phagocytic response (20, 56), we noted that, when these aggregates were bound to matrix, some appeared as large as 1 μm or greater (Ref. 21 and Figs. 3 and 5 herein). Thus, as in phagocytosis, the actin-myosin cytoskeleton may be required for the extension of plasma membrane processes around these large matrix-bound aggregates (56).

The internalization of retained and aggregated lipoproteins, however, has an additional element, namely avid surface binding, that does not exist with the phagocytosis of large particles in solution. Thus, additional actin-myosin-mediated processes may be needed to help “pry” the lipoproteins away from the matrix. For example, actin might be involved as both a scaffold for myosin to allow force generation per se as well as in anchoring the cells through focal adhesion complexes and podosomes (60), which would be necessary to transmit the force generation into successful separation of lipoproteins from the matrix. Other possible roles of the cytoskeleton that may be important in the engagement, internalization, and degradation of retained and aggregated LDL include facilitation of cellular motility, filopodia formation, and/or polarized lamellipod extension and retraction.

The precise actin and myosin signaling pathways involved in the uptake of matrix-retained lipoproteins represents another important goal of future studies. The use of pharmacological inhibitors can be extremely useful, and they have been employed extensively to study cytoskeletal signaling pathways, but some of these compounds may have effects on cells that are not directly related to the target molecules of interest. Transfecting cells with dominant negative constructs can help in this regard, but because macrophages are extremely difficult to transfect (Ref. 61 and our own observations), the utilization of this strategy in the experimental system described here represents a substantial technical challenge. Nonetheless, the current study provides evidence suggesting important roles for one or more members of the Rho family of GTPases, other than Rho itself, as well as for tyrosine kinase, PI3K, and myosin ATPase activities. Of interest are the findings by others that inhibition of Rac, as opposed to inhibition of Rac1 and Cdc42, enhances macrophage spreading, monocyte adherence to matrix, and phagocytosis of apoptotic cells by macrophages (62–64). These reports of the differential effects of Rac versus Rac1 and Cdc42 are consistent with our data showing their differential effects in the uptake and degradation of retained and aggregated LDL (Fig. 6).

The only compound used in this study that did not show a selective effect on the uptake and degradation of retained and aggregated LDL versus non-retained LDL was ML-7 (Fig. 8B), which blocks myosin II action by inhibiting myosin light chain kinase activity (54). In contrast, inhibition of myosin ATPase activity by BDM, a broad spectrum myosin ATPase inhibitor (55), was relatively selective in its effect (Fig. 8A). Thus, one or more myosin subtypes other than myosin II may play a specific role in the uptake and degradation of matrix-retained and aggregated LDL. In this light, Swanson et al. (59) have recently shown that myosins IC, V, and IXb are present in the phagosomes of macrophages that engage IgG-opsonized erythrocytes. Whether these myosins or other subtypes play a role in the interaction of macrophages with matrix-retained and aggregated LDL remains to be determined.

The complete identification of cell-surface molecules involved in the uptake of retained and aggregated LDL will be yet another important goal of future studies. Using RAP and an anti-LRP antibody, we show that LRP, which is present on lesional macrophages (65), plays a partial role. In reality, LRP may have a somewhat larger involvement than is evident from these data, because the phagocytic-like uptake of retained and aggregated LDL almost certainly involves multivalent interactions, and such interactions are known to be poorly competed by monovalent inhibitors (66). Interestingly, murine macrophage LRP has been shown to recognize native non-retained LDL (34), and our data are consistent with this finding (Fig. 2D). Moreover, the uptake of non-retained vortex-aggregated LDL by human vascular smooth muscle cells is also mediated by LRP (35), and we showed that the degradation of non-retained SMase-aggregated LDL by mouse peritoneal macrophages is substantially blocked by RAP (Fig. 2D). These data indicate that the recognition of aggregated LDL by macrophage LRP is not dependent on matrix retention. Nonetheless, when LRP contacts aggregated LDL that is retained on matrix, signaling through the cytoplasmic tail of the receptor, perhaps induced by receptor aggregation, may play a role in cytoskeletal signaling pathways. In this regard, LRP has been implicated in cellular signaling pathways involving protein phosphorylation (35, 67–70).

The fact that uptake is normal in LDL receptor-deficient...
Degradation of Matrix-retained LDL by Macrophages

FIG. 9. Model of the interaction of macrophages with matrix-retained and aggregated LDL. The data from this report and our previous studies have identified three continuous and sequential phases occurring during the interaction of cultured macrophages with matrix-retained and aggregated LDL. In Phase I, an initial period of prolonged cell-surface contact between the macrophage cell surface and the retained lipoproteins leads to partial selective CE uptake and hydrolysis (22). In Phase II, the macrophages internalize and degrade the lipoproteins, which partially involves LRP. This phase requires actin-myosin cytoskeletal processes, such as filopodia extension to facilitate cellular engulfment of the lipoproteins, force generation to "pry" the lipoproteins from the matrix, and/or cell motility. In Phase III, enough lipoprotein-cholesterol has been delivered to the macrophage to stimulate ACAT (9), and progressive CE accumulation ensues, leading to foam cell formation (21). See text for details.

macrophages indicates that either the LDL receptor is not involved or that in its absence, one or more other receptors can compensate for its absence. Scavenger receptors are also not involved, suggesting that cell-mediated oxidation of the retained LDL is not a major process or that it accounts for only a small proportion of internalized and degraded lipoproteins. In this context, Kaplan and Aviram (71) have recently reported that J774 macrophages internalize and degrade matrix-bound oxidized LDL (OxLDL). Degradation of retained OxLDL, however, required macrophage "activation" by phorbol esters, and the absolute amount of OxLDL degradation even after 18 h of incubation was very small compared with the degradation of retained and SMase-aggregated LDL in our study. Whether this distinction is due to methodological differences between the two studies or to true differences between retained, oxidized LDL versus retained, SMase-aggregated LDL will require further investigation.

Together with our previous studies, the current work indicates that a set of specific cellular events and processes come into play when macrophages interact with matrix-retained and aggregated LDL. We have begun to think of these events occurring in three continuous and sequential phases (Fig. 9). In Phase I, there is initial prolonged cell-surface contact between the macrophage cell surface and the retained lipoproteins, leading to partial selective CE uptake and hydrolysis (22). In Phase II, the macrophages internalize and degrade the lipoproteins, and this critical process partially involves LRP and actin-myosin cytoskeletal processes as discussed above. In Phase III, enough lipoprotein-cholesterol has been delivered to the macrophage to stimulate ACAT (9), and progressive CE accumulation ensues, leading to foam cell formation (21). Note that many of the processes described in Phases I and II would have been missed in the typical cell-culture experimental system, which emphasizes receptor-mediated endocytosis. Based on previous observations of the state of lipoproteins in actual atherosclerotic lesions, we propose that the phases described above closely resemble those occurring during foam cell formation in vivo. In this light, our ultimate goal is to study atherosclerotic foam cell formation in vivo using animal models in which some of the molecular events described herein are genetically altered.

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The Uptake and Degradation of Matrix-bound Lipoproteins by Macrophages Require an Intact Actin Cytoskeleton, Rho Family GTPases, and Myosin ATPase Activity

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