Retro-endocytosis of Low Density Lipoprotein by Cultured Human Skin Fibroblasts

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A fraction of the low-density lipoprotein (LDL) internalized by cells via receptor-mediated endocytosis follows a short-circuit pathway, termed "retro-endocytosis," that results in the rapid exocytosis of ligand. Results from the current study suggest that retro-endocytosis of LDL in human fibroblasts is caused by resurfacing of endocytotic vesicles that contain both free and receptor-bound ligand, resulting in discharge of vesicular contents and in spontaneous dissociation of LDL from its receptor. The bulk of the released LDL particles had the same size, density, and immunogenic properties as native LDL, indicating that they were discharged intact. Some of the retro-endocytosed LDL was larger than native LDL, and some exhibited altered sedimentation properties. When fusion of endosomes with lysosomes was inhibited by chilling cells to 18°C, the proportion of intracellular LDL subsequently released was unaffected, suggesting that retro-endocytosis does not require lysosomal participation. Furthermore, the shorter the internalization phase, the greater was the proportion of LDL subsequently released, suggesting that LDL was discharged from compartments formed early in endocytosis. Retro-endocytosis of LDL was stimulated by agents that neutralize acid intracellular compartments, such as ionophores (monensin) and weak bases (chloroquine and methylamine). Monensin increased the proportion of intracellular LDL released, suggesting that it had a direct effect on retro-endocytosis. The effect of weak bases appeared to be secondary to their ability to promote cellular accumulation of undegraded LDL. Thus, retro-endocytosis of LDL becomes a major pathway when intracellular compartments fail to maintain a low pH or where the intracellular concentration of LDL reaches abnormal levels. (Arteriosclerosis 5:45–54, January/February 1985)
tents are usually delivered to lysosomes except in certain specialized cell types where delivery to storage granules (fetal yolk sac), Golgi elements (neuroblastoma cells), or across cells (neonatal intestinal epithelial cells) has been observed. Recent evidence has shown that a variety of ligands internalized by receptor-mediated endocytosis follow a short circuit path that does not lead to degradation but results in the rapid exocytosis of intact ligand.

The occurrence of exocytosis in cultured smooth muscle cells and fibroblasts as well as isolated hepatocytes, reticulocytes, and macrophages suggests that retro-endocytosis may be a general phenomenon.

We have previously shown that retro-endocytosis is a rapid, temperature-dependent process that occurs following the binding and internalization of LDL by specific cell-surface receptors. The present study is focused on the mechanism(s) of retro-endocytosis and, in particular, on the following questions: (1) Is the LDL structurally altered during retro-endocytosis? (2) From which intracellular compartment is the internalized LDL released? (3) Can retro-endocytosis of LDL be modulated by agents that affect vesicular transport processes? Results suggest that most of the released LDL particles were structurally similar to native LDL. Receptor-ligand complexes, as well as free ligand, appear to be cycling from prelysosomal intracellular compartiments to cell-surface locations. Flux through this short-circuit pathway is facilitated by agents such as chloroquine and methylamine that induce excessive intracellular accumulation of undegraded LDL, as well as by agents like monensin that raise the pH of endosomes.

Methods

Cells and Lipoproteins

Human skin fibroblast strains previously characterized as LDL-receptor-normal (GM 3348) and LDL-receptor-internalization-defective (GM 2408A) were obtained from the Human Genetic Mutant Cell Repository (Camden, New Jersey). In addition, skin fibroblasts were established from a skin biopsy of a normocholesterolemic subject and were confirmed as LDL-receptor-normal. The occurrence of exocytosis in cultured smooth muscle cells and fibroblasts suggests that retro-endocytosis may be a general phenomenon.

We have previously shown that retro-endocytosis is a rapid, temperature-dependent process that occurs following the binding and internalization of LDL by specific cell-surface receptors. The present study is focused on the mechanism(s) of retro-endocytosis and, in particular, on the following questions: (1) Is the LDL structurally altered during retro-endocytosis? (2) From which intracellular compartment is the internalized LDL released? (3) Can retro-endocytosis of LDL be modulated by agents that affect vesicular transport processes? Results suggest that most of the released LDL particles were structurally similar to native LDL. Receptor-ligand complexes, as well as free ligand, appear to be cycling from prelysosomal intracellular compartments to cell-surface locations. Flux through this short-circuit pathway is facilitated by agents such as chloroquine and methylamine that induce excessive intracellular accumulation of undegraded LDL, as well as by agents like monensin that raise the pH of endosomes.

Binding, Internalization, Retro-endocytosis and Degradation of $^{125}$I-LDL

The retro-endocytosis of $^{125}$I-LDL was determined by using a pulse-chase procedure as previously described. Before each experiment, the number of LDL receptors was up-regulated by incubation of cells with culture medium containing 10% human LPDS. The cell layers then received lipoprotein-deficient culture medium containing the indicated concentration of $^{125}$I-LDL and were incubated at 37°C for the indicated time (pulse period). When pulse incubations were performed at 4°C, the medium was buffered with 10 mM N-2-hydroxy-ethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.4. The cells were chilled on ice and each layer was washed four times with 0.2% bovine serum albumin in phosphate-buffered saline (PBS) pH 7.4, and three times with PBS at 4°C. Surface-bound LDL was removed by incubation of the cell layers with PBS containing 4 mg/ml sodium heparin (Sigma) or 4 mg/ml dextran sulfate at 4°C for 45 to 60 minutes. The cells were rinsed three times with PBS and then incubated for up to 2 hours at 37°C in medium containing 10% LPDS and the indicated concentration of unlabelled LDL (chase period). Where specified, the cells were chase-incubated with serum-free medium containing 0.2% albumin without unlabelled LDL. The chase period culture medium was retained and the cells were washed three times with PBS at 4°C. The $^{125}$I-radioactivity (intracellular LDL after chase) and protein content were then determined in NaOH-solubilized cell layers. The release of $^{125}$I-protein and $^{125}$I-degradation products from cells was determined after incubation of the chase medium with an antibody to LDL (see immunoprecipitation of $^{125}$I-protein). As indicated, 30 μM chloroquine (Sigma), 5 mM methylamine (Sigma), and/or 25 μM monensin (Calbiochem-Behring Corporation) were used. In experiments where monensin dissolved in ethanol was used, 0.25% ethanol was added to dishes not receiving monensin. Unless indicated otherwise, each data point for the figures and tables represents the mean of determinations on duplicate dishes.

Immunoprecipitation of $^{125}$I-Protein

Sheep immune serum raised against human LDL was prepared as described by Albers et al. Gel diffusion studies indicated that this antibody did not react with HDL (free of apoprotein B), apoproteins A-I, A-II, C-I, C-II, C-III, D, E, or the d > 1.21 g/ml plasma fraction. Immunoprecipitation of $^{125}$I-protein was performed by adding 0.2 ml sheep immune serum to 1 ml chase medium containing 35 to 50 μg/ml carrier LDL protein followed by incubation at 37°C for 1 hour and then 4°C for 18 to 24 hours. This proportion of LDL to immune serum gave maximum precipitation. The immune precipitate was isolated by centrifugation, washed by recentrifugation in PBS, and counted for $^{125}$I. Trichloroacetic acid (TCA; final concentration of 10%), was added to the super-all.
The extent of retro-endocytosis was determined as the amount of 125I-radioactivity precipitated by an antibody to LDL. Immunoprecipitation of chase medium using affinity purified rabbit antihuman LDL gave similar results. The "retro-endocytosis index" is defined as the ratio of immunoprecipitable 125I-protein released during the chase to the intracellular content of 125I before the chase. As the total 125I in the cell at the end of the pulse would represent both 125I in the form of native LDL (immunoprecipitable) as well as some 125I that is partially or completely degraded (may not be immunoprecipitable), the retro-endocytosis index would represent a minimum value.

Immunological studies revealed that, although approximately 95% of the 125I-protein material present during the pulse incubation reacted with an antibody to LDL, only 50% to 70% of that released upon subsequent incubation in isotope-free chase medium was immunoreactive. Particles released during the chase that did not react with anti-LDL immune serum were separated from particles that had the same size and immunogenic properties as native LDL by gel filtration (Biogel A-5m; see molecular sieve chromatography). Autoradiographic analysis of sodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns of nonimmunoreactive 125I-particles purified by gel filtration yielded molecular weight species that appeared identical with apolipoproteins A-I and C-I through C-III. Isopycnic sucrose density gradient centrifugation indicated that these nonimmunoreactive particles exhibited a density of greater than 1.12 g/ml. These particles were found to be present in the 125I-LDL preparation (<1%). It appears that the release of nonimmunoreactive 125I-particles was the result of reversible binding to the cell surface of a radiolabeled HDL-like particle present in the original 125I-LDL preparation.

**Sedimentation Velocity Centrifugation**

Ultracentrifugation of lipoprotein samples was carried out using a two-step KBr gradient. Samples containing 125I-protein in 2 ml medium containing 10% lipoprotein-deficient serum were adjusted to a density of 1.080 g/ml with solid KBr and layered beneath 15 ml of a 1.045 g/ml solution 125I. Tubes were centrifuged at 65,000 rpm for 5.5 hours at 10°C in a Sorval 865B vertical rotor. Gradients were fractionated by drainage from the bottom of the tube. Fractions of 14 drops were collected and assayed to determine the amount of immunoreactive 125I-protein by incubation with anti-LDL immune serum (recovery >95%).

**Molecular Sieve Chromatography**

Chromatography of samples was carried out on a Biogel A-5m column (200–400 mesh, 1.5 × 90 cm) (Biorad), equilibrated with 100 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 10 mM EDTA. Samples contained 125I-protein in medium containing 10% LPDS and 500 µg unlabeled LDL. A sample volume of 3 ml was applied and the column eluted with the above buffer at a flow rate of 6–8 ml/h. Fractions of 60 drops (approximately 2 ml) were collected and assayed to determine the amount of immunoreactive 125I-protein by incubation with anti-LDL antibody (recovery >90%). Blue dextran and 125I were used for the location of the void and total bed volume, respectively.

**Results**

To examine the possibility that structural modification of LDL could occur during retro-endocytosis, the physical properties of retro-endocytosed LDL were compared with those of native LDL. In these studies, fibroblasts were pulse-labeled with 125I-LDL at 37°C, exposed to heparin to remove surface-bound 125I-LDL, and subsequently incubated in isotope-free chase medium at 37°C. The extent of retro-endocytosis was determined as the amount of 125I-radioactivity present in the chase medium that was precipitated by an antibody to LDL. Molecular sieve chromatography demonstrated that approximately 75% of the immunoreactive 125I-particles released into the chase medium eluted at the same position as pulse 125I-LDL and heparin-released 125I-LDL (Figure 1). The remainder were larger than native LDL, eluting in the void volume. Sedimentation velocity centrifugation indicated that approximately 70% of the chase-released immunoprecipitable 125I-protein exhibited a distribution profile like that of native 125I-LDL. The remaining particles either were retained in the density 1.080 g/ml fraction at the bottom of the tube or exhibited a flotation velocity greater than LDL, floating to the top of the tube (Figure 2). It appears that different lipoprotein fractions were released from fibroblasts: 1) intact LDL, 2) higher molecular weight particles, and 3) particles with altered sedimentation properties.

Our earlier observations indicated that retro-endocytosis was a rapid, temperature-sensitive process that occurred following the binding of LDL by specific cell-surface receptors. Experiments in which heparin was used to release surface-bound LDL before the chase period supported the concept that exocytosis of LDL was taking place, rather than the dissociation of surface-bound LDL. To establish this point, the metabolism of 125I-LDL by a mutant fibroblast strain, previously characterized as LDL-receptor-internalization-defective (GM 2408A), was examined (Table 1). The inability of the mutant fibroblast strain to internalize receptor-bound LDL was evident from the low intracellular concentration and rate of degradation of LDL in relation to the amount bound. The LDL-receptor internalization-defective cell strain released less LDL than receptor-normal cells, even...
though the mutant strain bound the same or more LDL than the receptor-normal strain.

To obtain further support for the concept of LDL exocytosis, the effects of agents that perturb vesicular transport but do not substantially alter the binding of LDL to cell surface receptors were examined (Table 2). Weak bases were utilized to impair the delivery of LDL to lysosomes and increase organelle pH. Fibroblasts pulse-labeled with $^{125I}$-LDL for 18 hours in the presence of chloroquine (30 µM) or methylamine (5 mM) retro-endocytosed two- to fourfold more LDL than control cells loaded in the ab-

![Figure 1](image1.png)

**Figure 1.** Molecular sieve chromatography of retro-endocytosed $^{125I}$-LDL. After incubation for 48 hours in lipoprotein-deficient serum, human skin fibroblasts (2 × 75 cm flasks) were pulse-labeled for 4 hours at 37°C with 40 µg/ml $^{125I}$-LDL. Cells were chilled to 0°C, extensively washed, treated with heparin, and chase-incubated for 2 hours at 37°C in lipoprotein-deficient medium. To concentrate chase-released particles in as small a volume as possible, the timing of the pulse-chase procedure on the second flask was delayed and the chase medium from the first flask was collected for use on the second flask. $^{125I}$-protein particles in the pulse, heparin, and chase media were fractionated using a Biogel A-5 m column, and the amount of immunoprecipitable $^{125I}$-protein was determined as described in Methods.

![Figure 2](image2.png)

**Figure 2.** Sedimentation velocity centrifugation of retro-endocytosed $^{125I}$-LDL. The pulse-chase protocol outlined in the legend to Figure 1 was followed. Ultracentrifugation of $^{125I}$-protein particles in the pulse and chase medium was carried out as described in Methods. Fractions were collected and assayed to determine the amount of immunoprecipitable $^{125I}$-protein. Fraction 1 represents the most dense fraction (~1.080 g/ml), and fraction 40 represents the least dense (~1.045 g/ml).

![Figure 3](image3.png)

**Figure 3.** Effect of chloroquine on the size of retro-endocytosed $^{125I}$-LDL. The protocol in Figure 1 was followed. Fibroblasts (35 mm dishes) were pulse-labeled for 24 hours with 30 µM chloroquine (●) or for 4 hours without chloroquine (○). The cells were washed, treated with heparin, and subsequently chase-incubated at 37°C for 2 hours with (●) or without (○) chloroquine. The chase medium was analyzed by molecular sieve chromatography followed by immunoprecipitation of $^{125I}$-protein.
Table 1. Retro-endocytosis of $^{125}$I-LDL by Mutant Fibroblast Strains

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Cell strain</th>
<th>Degraded in pulse</th>
<th>Bound before chase</th>
<th>Retro-endocytosed in chase</th>
<th>Intracellular after chase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LDL-receptor-normal (GM 2408A)</td>
<td>4082 ± 99</td>
<td>379 ± 5</td>
<td>74 ± 8</td>
<td>2653 ± 31</td>
</tr>
<tr>
<td></td>
<td>LDL-receptor-internalization-defective (GM 2408A)</td>
<td>235 ± 16</td>
<td>327 ± 7</td>
<td>22 ± 1</td>
<td>130 ± 2</td>
</tr>
<tr>
<td>2*</td>
<td>LDL-receptor-normal (GM 3348)</td>
<td>2084</td>
<td>129</td>
<td>54</td>
<td>484</td>
</tr>
<tr>
<td></td>
<td>LDL-receptor-internalization-defective (GM 2408A)</td>
<td>66</td>
<td>175</td>
<td>27</td>
<td>96</td>
</tr>
</tbody>
</table>

The indicated fibroblast strains were pulse-labeled for 4 hours with 20 μg/ml $^{125}$I-LDL, treated with heparin, and chase-incubated for 30 minutes in lipoprotein-deficient medium containing 20 μg/ml unlabeled LDL. The pulse-medium was analyzed for its content of noniodide TCA-soluble $^{125}$I-degradation products. The $^{125}$I-radioactivity released by heparin and the intracellular content of $^{125}$I-radioactivity after the chase were also determined. The chase medium was analyzed for its content of immunoprecipitable $^{125}$I-protein. Data are mean values of duplicates (Expt. 2) or mean values ± SEM of quadruplicate dishes (Expt. 1).

*LDL receptor activity was up-regulated for 16 hours only.

To approach the question of whether LDL was being retro-endocytosed before or after the fusion of ligand-containing vesicles with lysosomes, the effect of temperature was studied. At temperatures below 20°C, ligands are internalized by endocytosis, but the fusion of endosomes with lysosomes is selectively inhibited.15 We have recently demonstrated16 in the case of LDL, that endocytosis and receptor recycling occur at temperatures (≤20°C) at which LDL degradation is blocked. To test whether retro-endocytosis can occur without lysosomal participation, fibroblasts were pulse-incubated at 37°C or 18°C with $^{125}$I-LDL for various lengths of time, and retro-endocytosis was monitored by reincubation at the same respective temperature for 2 hours (Figure 4). At 18°C, $^{125}$I-LDL degradation was inhibited (Figure 4 A), while intracellular accumulation still occurred, albeit at a slower rate than at 37°C (Figure 4 B). At both

Table 2. Effect of Lysosomotropic Inhibitors on Retro-endocytosis of $^{125}$I-LDL

<table>
<thead>
<tr>
<th>Pulse Time (hr)</th>
<th>Addition</th>
<th>Bound before chase</th>
<th>Retro-endocytosed in chase</th>
<th>Degraded in chase</th>
<th>Intracellular after chase</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>None</td>
<td>109 ± 8</td>
<td>75 ± 7</td>
<td>466 ± 20</td>
<td>2409 ± 117</td>
</tr>
<tr>
<td>18</td>
<td>Chloroquine, Methylamine</td>
<td>127 ± 4</td>
<td>303 ± 9</td>
<td>356 ± 10</td>
<td>11,318 ± 304</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>86 ± 2</td>
<td>302 ± 8</td>
<td>353 ± 8</td>
<td>2768 ± 40</td>
</tr>
</tbody>
</table>

Fibroblasts were pulse-labeled with 40 μg/ml $^{125}$I-LDL for the indicated times, treated with heparin and chase-incubated for 2 hours in lipoprotein-deficient medium containing 40 μg/ml unlabeled LDL. Where indicated, 30 μM chloroquine and 5 mM methylamine were employed. The $^{125}$I-radioactivity releasable by heparin and the intracellular content of $^{125}$I-radioactivity after the chase incubation were determined. The chase medium was analyzed for its content of immunoprecipitable $^{125}$I-protein and $^{125}$I-degradation products. Data are mean values ± SEM of the triplicate dishes.
Dependence of retro-endocytosis of $^{125}$I-LDL on time of exposure to $^{125}$I-LDL at 37° C and 18° C. Fibroblasts were pulse-labeled at 37° C (closed symbols) or 18° C (open symbols) for the indicated times with 40 µg/ml $^{125}$I-LDL, treated with heparin, and chase-incubated in lipoprotein-deficient medium containing 40 µg/ml unlabeled LDL for 2 hours at the same temperature used during the pulse incubation. When incubations were performed at 18° C, the medium was buffered with 10 mM HEPES (pH 7.4). The amounts of immunoprecipitable $^{125}$I-protein (circles) and $^{125}$I-degradation products (triangles, dotted line) released with the chase medium were determined and the cell layer was analyzed for intracellular $^{125}$I-radioactivity after the chase incubation. The intracellular content of $^{125}$I-radioactivity before the chase (squares) is the sum of the intracellular $^{125}$I-radioactivity remaining after the chase, plus immunoprecipitable $^{125}$I-protein, plus $^{125}$I-degradation products released during the chase. The retro-endocytosis index is the ratio of immunoprecipitable $^{125}$I-protein released in the chase to the intracellular content of $^{125}$I-radioactivity before the chase. Data in the inset is from a separate experiment in which cells were chase-incubated at 37° C for up to 4 hours.

Because rapid receptor recycling may present a possible mechanism by which cells could release LDL previously internalized by the LDL receptor pathway, we evaluated the effects of monensin on retro-endocytosis. Monensin has been shown to raise the pH of normally acid intracellular compartments, inhibit receptor recycling, prevent dissociation of receptor-ligand complexes within endosomes, and inhibit transfer of ligand from endosomes to lysosomes. That monensin was effective in reducing LDL receptor binding activity was confirmed by the fact that heparin-releasable $^{125}$I-LDL was reduced by 65% when fibroblasts were pulse-incubated for 30 minutes with 25 µM monensin (Table 3). The expected inhibition of LDL degradation was also observed (>90%). Despite a reduction in the amount of LDL endocytosed, the ionophore increased retro-endocytosis (60% to 200%). With cells loaded in the absence of monensin, its inclusion in the chase phase of the experiment alone was sufficient to increase both the amount of LDL released and the retro-endocytosis index. Even with cells loaded for 18 hours in the presence of chloroquine, the inclusion of monensin in the chase medium stimulated retro-endocytosis. This combined drug treatment dramatically increased the amount of LDL released (40-fold) and the retro-endocytosis index (10-fold).

One possible mechanism to explain retro-endocytosis is that a proportion of the internalized LDL-receptor complexes recycle to the cell surface where some of the LDL dissociates from its receptor and is released into the medium. Agents that block acidification of endocytic vesicles, such as monensin and chloroquine, may enhance the process by preventing intracellular LDL-receptor dissociation. To test this possibility, preloaded fibroblasts were incubated at 37° C in culture medium containing agents known to promote the dissociation of LDL from its receptor.
### Table 3. Effect of Monensin on Retro-endocytosis of 125I-LDL

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>125I-LDL (ng · mg⁻¹ cell protein)</th>
<th>Retro-endocytosed in chase (RE)</th>
<th>Degraded after chase (D)</th>
<th>Intracellular after chase (Iu)</th>
<th>Retro-endocytosis index (RE/Iu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse</td>
<td>Chase</td>
<td>Bound before chase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time (hr)</td>
<td>Addition</td>
<td>Time (hr) Addition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>None</td>
<td>0.5</td>
<td>None</td>
<td>324 ± 15</td>
<td>65 ± 1</td>
</tr>
<tr>
<td>Monensin</td>
<td>116 ± 11</td>
<td>104 ± 1</td>
<td>45 ± 1</td>
<td>915 ± 7</td>
<td>0.098</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>4</td>
<td>None</td>
<td>207 ± 20</td>
<td>131 ± 31</td>
</tr>
<tr>
<td>Monensin</td>
<td>386 ± 21</td>
<td>253 ± 6</td>
<td>4500 ± 179</td>
<td>0.075</td>
<td></td>
</tr>
<tr>
<td>Chloroquine</td>
<td>179 ± 18</td>
<td>1194 ± 17</td>
<td>17 ± 1</td>
<td>17,236 ± 393</td>
<td>0.064</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>4880 ± 184</td>
<td>152 ± 3</td>
<td>13,489 ± 208</td>
<td>0.263</td>
<td></td>
</tr>
</tbody>
</table>

The conditions outlined in Table 2 were employed. Fibroblasts not exposed to monensin (25 μM) received 0.25% ethanol.

### Table 4. Effect of Agents that Dissociate LDL-Receptor Complexes on Retro-endocytosis of 125I-LDL

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Addition</th>
<th>Retro-endocytosed LDL (ng/mg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse</td>
<td>Chase</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>121 ± 6</td>
</tr>
<tr>
<td>Dextran sulfate</td>
<td></td>
<td>293 ± 14†</td>
</tr>
<tr>
<td>Heparin</td>
<td></td>
<td>213 ± 5‡</td>
</tr>
<tr>
<td>EGTA</td>
<td></td>
<td>316 ± 24†</td>
</tr>
<tr>
<td>18 Chloroquine</td>
<td></td>
<td>344 ± 33</td>
</tr>
<tr>
<td>Chloroquine + dextran sulfate</td>
<td></td>
<td>1035 ± 67*</td>
</tr>
<tr>
<td>0.5 None§</td>
<td></td>
<td>65 ± 1</td>
</tr>
<tr>
<td>Dextran sulfate</td>
<td></td>
<td>205 ± 4†</td>
</tr>
<tr>
<td>0.5 Monensin</td>
<td></td>
<td>104 ± 1</td>
</tr>
<tr>
<td>Monensin + dextran sulfate</td>
<td></td>
<td>253 ± 5†</td>
</tr>
</tbody>
</table>

### Figure 5. Effect of Dextran Sulfate on the Retro-endocytosis of 125I-LDL

Fibroblasts were pulse-labeled with 40 μg/ml 125I-LDL for the indicated times, treated with heparin, and chase-incubated for 2 hours in serum-free medium containing 0.2% albumin. Where indicated, 30 μM chloroquine, 25 μM monensin, 4 mg/ml dextran sulfate, 4 mg/ml heparin, and 3 mM EGTA, pH 7.6, were employed. Other conditions were the same as those described in Table 3.

$p < 0.02; \hat{p} < 0.001.$

†Control value used for statistical evaluation (unpaired comparison) for each condition of the pulse incubation.

§Dishes contained 0.25% ethanol.

When cells were pulse incubated at 4°C, a temperature that allows binding but prevents internalization, the inclusion of dextran sulfate in the chase medium had no effect on the release of residual surface-bound LDL (Figure 5). In contrast, after loading at 37°C, retro-endocytosis was increased by the addition of dextran sulfate to the medium. Since the amount of LDL bound to the cells was approximately the same after the 37°C and 4°C loading periods, dextran sulfate must have stimulated release of LDL from within the cell, suggesting the return of LDL-receptor complexes to the cell surface.

The time-course of the reappearance of LDL at the cell surface was studied under conditions that dramatically increase retro-endocytosis, i.e., preloading in the presence of chloroquine and subsequent exposure to monensin during the chase phase (Figure 6). The amount of LDL on the cell surface was assayed by exposing fibroblasts to a second 4°C hep-
Retro-endocytosed Degraded
Heparin-releasable
(post-chase)

Figure 6. The increase in cell-surface $^{125}$I-LDL as a function of chase time under conditions that enhance retro-endocytosis. One set of fibroblasts were pulse-labeled with 5 $\mu$g/ml $^{125}$I-LDL for 18 hours in the presence of 30 $\mu$M chloroquine. Monensin (25 $\mu$M) was present during the final 15 minutes of the pulse period. The cells were treated with heparin containing 10% LPDS at 4°C and chase-incubated at 37°C for the indicated time in serum-free medium containing albumin with chloroquine and monensin as described in the Methods section. For the zero time value, cold-chase medium was added and immediately removed. The chase medium was analyzed for immuno-precipitable $^{125}$I-protein and $^{125}$I-degradation products (A). After the chase, the cells were washed once with PBS containing albumin, chilled, and retreated with heparin at 4°C for 1 hour. The medium was collected and analyzed for its content of $^{125}$I-radioactivity (B). The same procedure was followed using more fibroblasts, substituting unlabeled LDL for $^{125}$I-LDL. After the second treatment with heparin, the cells were chilled, washed three times with PBS containing albumin and then assayed for LDL-receptor-activity by incubation at 4°C with lipoprotein-deficient medium containing 5 $\mu$g/ml $^{125}$I-LDL and 10 mM HEPES (pH 7.4). The cells were washed seven times as described in Methods, and the $^{125}$I-radioactivity associated with NaOH-solubilized cell-layers measured (C).

arin treatment after the pulse-chase procedure. To estimate the relative number of receptors left on the cell surface after the chase, another group of cells was incubated with unlabeled LDL during the pulse-chase procedure, exposed to heparin at 4°C, and subsequently incubated with $^{125}$I-LDL at 4°C. Both the amounts of LDL retro-endocytosed into the chase medium (Figure 6 A), as well as the amount of $^{125}$I-LDL released during the postchase heparin treatment (Figure 6 B), increased rapidly within the first minutes of the chase. In contrast, the relative number of functionally active LDL receptors (Figure 6 C) decreased during the chase period, as would be expected if chloroquine and monensin were reducing the rate of receptor recycling. These data are consistent with the hypothesis that LDL-receptor complexes are returning from intracellular compartments to the cell surface.

Discussion

We have previously demonstrated that a fraction of the LDL internalized via receptor-mediated endocytosis escapes lysosomal degradation and instead enters a short-circuit pathway, termed "retro-endocytosis," which results in the rapid discharge of LDL from fibroblasts and smooth muscle cells. The present study was conducted to identify and characterize the cellular mechanism(s) involved in the retro-endocytosis of LDL and evaluate the changes in the physical properties of LDL processed by this pathway.

When the possibility that structural modification of LDL could occur during retro-endocytosis was examined, observations indicated that the bulk of the released LDL particles were structurally identical to native LDL, having the same size, density and immunogenic properties. However, some of the released LDL (approximately 20%) was larger than native LDL and some exhibited altered sedimentation properties, suggesting that cellular processing had occurred. It is not known if these two populations of altered LDL particles are related. Recently, Green-span and St. Clair showed that some of the LDL retro-endocytosed from monkey-skin fibroblasts had a greater density than the parent monkey LDL. In addition, they showed that the retro-endocytosed material was less immunoreactive with monkey LDL immune serum than native LDL. Our initial observations indicated that 30–50% of the $^{125}$I-protein released from human fibroblasts was not recognized by human LDL immune serum. Subsequent studies, however, demonstrated that the release of the non-immunoreactive $^{125}$I-particles was the result of reversible binding to the cell surface of a radiolabeled HDL-like particle present in the original $^{125}$I-LDL preparation (see Methods section).

Results with skin fibroblasts derived from a patient with familial hypercholesterolemia confirmed that retro-endocytosis of LDL is dependent upon the prior internalization of LDL by the LDL receptor. The mutant fibroblast strain, previously characterized as
LDL-receptor-internalization defective, released less LDL than receptor-normal fibroblasts, even though the mutant strain had a normal ability to bind LDL. These results support the concept that release is the expression of retro-endocytosis from intracellular sites rather than the dissociation of residual LDL from surface sites. When considering the intracellular compartment(s) involved, several lines of evidence indicate that retro-endocytosis does not require the participation of lysosomes. First, the particle does not appear to be degraded. Second, kinetic studies indicate that retro-endocytosis occurs rapidly, before LDL has reached the lysosomal compartment. Third, retro-endocytosis occurs at temperatures of 20°C or less, at which fusion of endosomes with lysosomes is inhibited. We have previously demonstrated that LDL endocytosis and receptor recycling continue to occur at temperatures of 20°C or less at which LDL degradation is blocked. Current results indicate that the proportion of intracellular LDL retro-endocytosed was similar at 37°C and 18°C. Furthermore, the shorter the internalization phase, the greater the proportion of LDL that was subsequently released, suggesting that LDL is released from endosomes formed early in the endocytic pathway. Finally, lysosomotropic inhibitors, agents known to block LDL degradation, enhanced the amount of LDL retro-endocytosed.

Insight into the cellular mechanism involved in retro-endocytosis was provided by studies using weak bases and ionophores, two classes of agents with similar modes of action. Both classes have been shown to raise the pH of normally acidic compartments and inhibit transfer of ligand from endosomes, may enhance the resurfacing process by increasing the relative proportion of vesicles in the endocytic pathway. Finally, lysosomotropic inhibitors, agents known to block LDL degradation, enhanced the amount of LDL retro-endocytosed.

In summary, results from the present study suggest that some of the LDL internalized by receptor-mediated endocytosis is rapidly returned to the cell surface from intracellular compartments formed early in the endocytic pathway (see Figure 7, Routes 1–3). Receptor-ligand complexes may recycle from endosomes that have not yet undergone marked acidi-
ification (Routes 1 and 2). Once the pH of the endosomes becomes acidic, LDL irreversibly dissociates from its receptor. The receptor then returns to the cell surface (Figure 7, Route 3) and the bulk of the LDL is transported to lysosomes for degradation (Figure 7, Route 4). A fraction of LDL may be returned to the cell surface free in vesicular fluid (Route 3). Recycling receptor-rich vesicles subsequently fuse with the plasma membrane, discharging their contents and allowing LDL to dissociate from its receptor. Some of the LDL may be modified during this recycling process. The physiological significance of retro-endocytosis of lipoproteins by cells remains to be elucidated. This process may be significant in atherosclerosis, since it could contribute to the delivery of LDL cholesterol to cells, and lead to chemical modification of LDL so that it is recognized by the "scavenger" receptor on macrophages. The present study indicates that retro-endocytosis becomes a quantitatively important alternate pathway in the cellular processing of LDL when the intracellular concentration of undegraded LDL reaches abnormal levels, as in the case of lysosomal deficiencies, or when normally acidic intracellular compartments fail to maintain a low pH.

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