Retroendocytosis of High Density Lipoproteins by the Human Hepatoma Cell Line, HepG2

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When human HepG2 hepatoma cells were pulsed with \(^{125}\)I-labeled high density lipoproteins (HDL) and chased in fresh medium, up to 65% of the radioactivity released was precipitable with trichloroacetic acid. Cell-internalized \(^{125}\)I-HDL contributed to the release of acid-precipitable material; when cells were treated with trypsin before the chase to remove \(^{125}\)I-HDL bound to the outer cell membrane, 50% of the released material was still acid-precipitable. Characterization of the radioactive material released by trypsinized cells revealed the presence of particles that were similar in size and density to mature HDL and contained intact apolipoproteins (apo) A-I and A-II. The release of internalized label occurred at 37°C but not at 4°C. Monensin, which inhibits endosomal recycling of receptors, decreased the binding of \(^{125}\)I-HDL to cells by 75%, inhibited the release of internalized radioactivity as acid-precipitable material by 80%, and increased the release of acid-soluble material by 90%. In contrast, the lysosomal inhibitor chloroquine increased the association of \(^{125}\)I-HDL to cells by 25%, inhibited the release of precipitable material by 10%, and inhibited the release of acid-soluble radioactivity by 80%. Pre-incubation with cholesterol caused a 50% increase in the specific binding, internalization, and resecretion of HDL label. Cholesterol affected the release of acid-precipitable label much more (+90%) than that of acid-soluble material (+20%). Taken together, these findings suggest that HepG2 cells can bind, internalize, and resecrete HDL by a retroendocytic process. Furthermore, the results with cholesterol and monensin indicate that a regulated, recycling, receptor-like molecule is involved in the binding and intracellular routing of HDL. (Arteriosclerosis 10:582-590, July/August 1990)

High density lipoproteins (HDL) stimulate the removal of cholesterol from several types of peripheral cells in culture and are thought to play a similar role in vivo. They have, therefore, been implicated in the transport of cholesterol from peripheral tissues to the liver for disposal or re-utilization, a process called reverse cholesterol transport. Such a role for HDL is consistent with the negative correlation observed between its serum concentration and the incidence of cardiovascular disease.

In species with cholesteryl ester transfer protein, HDL cholesterol can be transported to the liver via transfer of cholesteryl esters to chyomicrons or very low density lipoproteins, whose remnants can be taken up by the liver. Alternatively, HDL itself can interact directly with the liver, presumably via a specific binding protein, which may mediate the uptake and degradation of the lipoprotein by a pathway similar to the endocytosis of low density lipoproteins (LDL) and remnant particles.

However, the interaction of HDL with liver cells is more complex than that of LDL. Studies with whole animals have shown that more cholesteryl esters than HDL apolipoproteins are taken up. This preferential uptake does not correlate with high-affinity binding of HDL, which does not require internalization of the lipoprotein, and may be enhanced by cholesteryl ester transfer protein. HDL has also been shown to mediate the efflux of cholesterol from hepatocytes. As in peripheral cells, this process is associated with specific high-affinity binding of HDL, but in contrast to the binding of LDL, it does not necessarily lead to degradation of the lipoprotein. It has, therefore, been referred to as reversible binding by Bachorik et al.

In the present experiments, we studied the interaction of HDL with cells of the human hepatoma cell line, HepG2. These cells display the morphological and biosynthetic characteristics of liver parenchymal cells and were, therefore, used as a model for human hepatocytes. We confirmed that these cells can bind, internalize, and degrade \(^{125}\)I-HDL. However, results obtained using a pulse-chase protocol suggest that a substantial proportion of the internalized \(^{125}\)I-HDL is re-secreted intact. Furthermore, studies with cholesteryl and monensin indicate that a regulated receptor-like factor that recycles through the endosomal compartment of the cell is involved in the handling of HDL.

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Paul D. Roach was a post-doctoral fellow of the Canadian Heart Foundation. This work was supported by the National Health and Medical Research Council of Australia and the Flinders Medical Center Research Foundation.

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Received March 20, 1989; revision accepted February 20, 1990.
Methods

Materials

The human hepatoma cell line, HepG2, was obtained from the American Tissue Culture Collection. Minimum essential medium (MEM), fetal calf serum (FCS), and 0.05% (wt/vol) trypsin in 0.02% (wt/vol) ethylenediamine-tetraacetate (EDTA) were from Flow Laboratories (Irvine, UK). Culture flasks were from Nunc (Roskilde, Denmark). Bovine serum albumin (BSA), chloroquine, chloramphenicol, cholesterol (99%+), gentamicin, and monensin were from Sigma Chemical Company (St. Louis, MO). Sodium 125Iiodide was from Amersham Australia (Adelaide, SA).

Isolation of Lipoproteins and Lipoprotein Deficient Plasma

Plasma was prepared from fresh human blood anticoagulated with EDTA (1 mg/ml). Lipoproteins, LDL (1.019<d<1.055 g/ml), HDL (1.07<d<1.21 g/ml), and HDL3 (1.21<d<1.21) were prepared by sequential preparative ultracentrifugation including a wash to remove albumin. The lipoproteins were dialyzed overnight against phosphate-buffered saline (PBS) and stored at 4°C in the presence of chloramphenicol (50 mg/ml) and gentamicin (100 mg/ml). Human lipoprotein deficient plasma (LPDP) was recovered after ultracentrifugation at d=1.25 g/ml and was stored frozen (-70°C) after heat inactivation of complement and lecithin cholesterol acyl transferase (LCAT) activity.

Radioiodination of High Density Lipoprotein

HDL and HDL3 were radioiodinated by the iodine monochloride technique as modified for lipoproteins and were filter sterilized with a 0.45 μm Millipore filter. On average, 96% of the radioactivity was precipitable in 12% trichloroacetic acid, and 4% was associated with lipids. After electrophoresis through a 10% polyacrylamide gel containing 1% sodium dodecyl sulfate (SDS), two major protein bands, corresponding to apolipoproteins (apo) A-I and A-II, were seen. HDL, but not HDL3, displayed in the apo E region a minor band which contained 0.4% of the radioactivity associated with HDL protein. Labeled HDL and HDL3 were used within 2 weeks of preparation.

Cell Cultures

The HepG2 cells were maintained essentially as described by others. Cells were treated with 0.05% (wt/vol) trypsin in 0.02% (wt/vol) EDTA for 10 minutes at 37°C to detach them from the dishes; they were then split 1:3 into T-25 cm² flasks with 5 ml of MEM containing 10% (vol/vol) heat-inactivated FCS and were incubated for 2 days to allow them to adhere and grow to 85% confluency. Before use, the cells were washed three times with PBS (5 ml/wash) and were incubated in fresh MEM containing 5% LPDP for 24 hours at 37°C in 5% CO2/air. After this time, the monolayers were confluent and ready for incubation with labeled HDL.

Binding and Internalization Studies

Confluent cell monolayers were washed three times with PBS just before the addition of fresh MEM containing 5% LPDP and the desired amount of 125I-HDL or 125I-HDL3. In some experiments, a 20-fold excess of unlabeled HDL was added to determine the amount of radioactivity specifically associated with the cells. Before removal of the cells from the flasks with 0.1 M NaOH for counting of radioactivity and assaying of protein, the cells were washed extensively to remove loosely adsorbed radioactivity; twice each with 5 ml of ice-cold PBS (pH 7.4), 5 ml of ice-cold PBS containing 0.2% BSA, and finally with 5 ml of ice-cold PBS. This is the protocol referred to whenever the cells are said to be washed.

The experiments were carried out at 4°C to study binding only and at 37°C to determine the total cell association (binding plus internalization). To estimate the amount of radioactivity internalized, the cells were first washed, treated with 0.05% (wt/vol) trypsin in 0.02% (wt/vol) EDTA for 10 minutes at 37°C. They were pelleted by low-speed centrifugation and then washed extensively as above by resuspension and centrifugation. Duplicate or triplicate dishes were used for every determination.

Pulse-Chase Studies

Pulse-chase studies were carried out by using 100 μg/ml of 125I-HDL or 125I-HDL3 in MEM/5% LPDP. Dishes containing confluent monolayers of HepG2 cells were incubated with radiolabeled HDL at 37°C for the desired time. Under sterile conditions, the medium was removed and the cells were washed. Fresh MEM containing 5% LPDP was added to the dishes, and the cells were chased. To study the release of internalized material only, the cells were washed, treated with 0.05% (wt/vol) trypsin in 0.02% EDTA for 10 minutes at 37°C, washed by centrifugation, incubated in fresh MEM/5% LPDP in Nunc 10 ml flat-sided tubes, and chased. After the desired time, the tubes were centrifuged and the medium was removed. An equal volume of 24% trichloroacetic acid (TCA) was added to aliquots of the medium to precipitate the protein, with BSA (5 mg/ml) added as carrier. After centrifugation at 10 000 g for 15 minutes, the radioactivity in the TCA-precipitable (pellet) and TCA-soluble (supernatant) fractions was measured. Cells attached to the tubes and pelleted during centrifugation were solubilized in 0.1 M NaOH for counting of radioactivity and determination of protein. The total of the radioactivity found in the media plus that associated with the cells after the chase was taken to be the radioactivity associated with the cells before the chase. In some experiments, the trypsinized cells were allowed to re-attach to the dishes (5 hours in fresh media) before starting the chase incubation. Duplicate or triplicate dishes were used for every determination.

Treatment of Cells

Some cells were incubated in the presence of 50 μg/ml cholesterol for 24 hours before incubating with 125I-HDL3 for binding, internalization, and chase studies. Some cells, including some of the cells incubated with cholesterol, were incubated in the presence of 25 μM monensin.
and 100 μg/ml unlabeled HDL for 2 hours before incubating with labeled HDL. Control cells were exposed to 100 μg/ml of unlabeled HDL only during this time. The monensin was also present during the pulse and chase incubations. In a second type of monensin experiment, the cells were only exposed to the ionophore during the chase incubation. Other cells were incubated with 100 μM chloroquine for 2 hours before incubating with 125I-HDL as well as during the pulse and chase incubations.

Analysis of Internalized and Released Radioactivity

The radioactive material inside the cells was characterized as follows. The cells were pulsed with 125I-HDL, were washed, treated with 0.05% (wt/vol) trypsin in 0.02% (wt/vol) EDTA for 10 minutes at 37°C, were washed and then were homogenized in water with 20 strokes of a Dounce homogenizer. The homogenate was adjusted to a density of 1.21 g/ml with KBr and subjected to ultracentrifugation for 36 hours at 100,000 g in a T1 50.3 Beckman rotor. The radioactive material that floated was subjected to electrophoresis on 2.5% to 27% concave gradient polyacrylamide gels to analyze by electrophoresis on 10% polyacrylamide gel containing SDS and was followed by autoradiography and densitometric scanning.

Results

In preliminary experiments, the association of 125I-HDL with HepG2 cells at 37°C increased linearly with time, essentially attaining equilibrium within 2 hours. The association demonstrated specificity for HDL in that it was reduced by 60% in the presence of a 20-fold excess of unlabeled HDL, while a similar excess of unlabeled LDL displaced less than 10% of the labeled HDL. The association also exhibited saturation kinetics with increasing concentrations of 125I-HDL essentially as published by Dashti et al.13 Scatchard analysis revealed a curvilinear plot, which was consistent with the presence of a high-affinity, low-capacity site and a low-affinity, high-capacity site. The Kd of the high-affinity site was 40 μg/ml. The concentration of 100 μg/ml 125I-HDL was chosen for the subsequent studies because the high-affinity sites were just achieving saturation and the low-affinity sites were still a minor component of the binding.

The metabolism of the cell-associated 125I-HDL was studied in a series of pulse-chase experiments. In the first experiment, HepG2 cells were incubated with 125I-HDL for 24 hours and were subsequently incubated in fresh medium. The cells progressively released radioactive material so that 40% of the radioactivity that had become associated with the cells was recovered in the chase medium after 3 hours. However, at any time during the 3-hour chase, 60% to 65% of the released radioactivity was precipitable with 12% TCA (Figure 1). The proportion of TCA-precipitable material released during the 3-hour chase period remained constant when the length of the pulse incubation was varied between 3 and 24 hours (data not shown).

To establish whether cell-internalized 125I-HDL was released as TCA-precipitable radioactivity, the cells were treated with trypsin before chasing. As shown in Table 1, trypsinization removed 60% of the cell-associated radioactivity. When chased at 37°C immediately after trypsinization, the cells released 33% of their label, 50% of which was acid-precipitable. At 4°C, these cells released only 1.7% of their radioactivity, indicating that the trypsin treatment effectively removed 125I-HDL from the outer plasma membrane; untreated cells released 12% of their radioactivity at 4°C. In these experiments, the cells were all detached at the start of the chase, with 70% attaching to the dishes during the 3-hour incubation. In other experiments, trypsinized cells were incubated for 5 hours in fresh media to allow more than 95% of them to re-attach to the dishes before starting a 3-hour chase period in new fresh media. During the 3-hour chase, 63% of the material released by the attached cells was TCA-precipitable.

To evaluate the trypsin protocol, cells were pulsed with 125I-HDL for 2 hours at 4°C in the presence of 5 mM NaN3 to prevent the internalization of 125I-HDL. They were then washed, incubated for 10 minutes at 37°C in the presence or absence of 0.05% (wt/vol) trypsin in 0.02% EDTA (wt/vol), and washed before counting. The protease treatment proved very effective; treated cells retained less
Table 1. Effect of Trypsin on Release of High Density Lipoprotein Into Chase Medium

<table>
<thead>
<tr>
<th>Cells</th>
<th>Cell-associated before chase</th>
<th>Released into chase medium</th>
<th>TCA-precipitable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chased at 4°C</td>
<td>220±15</td>
<td>26±3</td>
<td>24±2</td>
</tr>
<tr>
<td>Chased at 37°C</td>
<td>220±15</td>
<td>105±9</td>
<td>70±4</td>
</tr>
<tr>
<td>Trypsin treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chased at 4°C</td>
<td>90±4</td>
<td>1.5±0.3</td>
<td>1.4±0.2</td>
</tr>
<tr>
<td>Chased at 37°C</td>
<td>90±4</td>
<td>29±1</td>
<td>15±2</td>
</tr>
</tbody>
</table>

Values are given in ng HDL/mg cell protein and are radioactivity counts divided by the specific radioactivity of $^{125}$I-HDL. They are the means±SEM of triplicate determinations. HepG2 cells were incubated with $^{125}$I-HDL for 2 hours at 37°C, were washed, incubated in the presence or absence of 0.05% (wt/vol) trypsin in 0.02% (wt/vol) EDTA for 10 minutes at 37°C, and were washed and chased for 3 hours at 37°C. HDL = high density lipoprotein, EDTA = ethylenediaminetetraacetate, TCA = trichloroacetic acid.

Release of TCA-precipitable material released by trypsin-treated cells was analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Two protein bands were found to contain radioactivity, and their apparent molecular weights corresponded to those of apo A-I (28 000) and apo A-II (17 000) of tracer $^{125}$I-HDL. Densitometric scanning of the autoradiograms revealed that the apo A-I band contained 73% of the radioactivity and the apo A-II band, 27%, compared to 50% for the apo A-I and 48% for the apo A-II bands of $^{125}$I-HDL not exposed to cells.

The nature of the radioactive material internalized and released by trypsinized cells was also investigated by ultracentrifugation and gradient gel electrophoresis. About 67% of the trypsin-resistant label and 55% of that released during the chase floated at d=1.21 g/ml. Gradient gel electrophoresis, autoradiography, and scanning densitometry revealed that the migration of both the internalized and resecreted labeled moieties was similar to that of marker $^{125}$I-HDL (Figure 2).

Monensin was used to investigate the possible involvement of endosomal recycling in the binding, internalization, and resecretion of $^{125}$I-HDL. The carboxylic ionophore had a pronounced inhibitory effect on the association of $^{125}$I-HDL to cells pre-incubated in its presence; the treated cells contained 70% less radioactivity than control cells (Table 2). Furthermore, the monensintreated cells released proportionally less acid-precipitable material during the 3-hour chase period. Precipitable material accounted for only 39% of the radioactivity released by treated cells compared to 72% for control cells. These cells were not treated with trypsin before the chase.

In another type of experiment, trypsinized cells were exposed to monensin during the chase only. There was no difference in the amounts of radioactivity released by the cells into the chase medium, but the composition differed markedly. In the presence of monensin, only 8% of the radioactivity released was acid-precipitable in contrast to 50% for control cells (Figure 3).

Chloroquine had totally different effects. The lysosomal inhibitor did not inhibit the association of $^{125}$I-HDL with the cells; treated cells contained 26% more radioactivity than control cells (Table 3). It also had little effect on the release of TCA-precipitable material, decreasing it by only 12%. However, it did decrease the release of acid-soluble degradative products by 80%. Consequently, the proportion of precipitable material increased from 64% to 88%. These cells were not treated with trypsin before the chase, but similar results were obtained when they were; the amount of radioactivity internalized by the cells was increased by 24%, the release of TCA-precipitable material was decreased by 16%, and the degradation was decreased by 50%.

When HepG2 cells were pre-incubated with cholesterol for 24 hours before the pulse incubation with $^{125}$I-HDL at 4°C in the presence or absence of unlabeled HDL, a 50% increase in specific binding was observed (Table 4). In parallel cultures at 37°C, the internalization of $^{125}$I-HDL and the release of internal...
Trypsin in 0.02% (wt/vol) EDTA for 10 minutes at 37°C, and were the means±SEM of triplicate determinations. HepG2 cells were incubated in the presence of 100 μg/ml unlabeled high density lipoprotein (HDL) and in the presence or absence of 25 μM monensin for 2 hours at 37°C. Cells were then washed, pulsed with 100 μg/ml 125I-HDL for 2 hours at 37°C in the presence or absence of 25 μM monensin, and were washed and chased in fresh medium for 3 hours at 37°C in the presence or absence of 25 μM monensin.

Table 2. Effect of Monensin on Association and Release of High Density Lipoprotein

<table>
<thead>
<tr>
<th>Cells</th>
<th>Cell-associated in chase medium</th>
<th>TCA-precipitable in chase medium</th>
<th>TCA-soluble in chase medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>336±34</td>
<td>109±3</td>
<td>43±2</td>
</tr>
<tr>
<td>Monensin</td>
<td>105±5</td>
<td>12±2</td>
<td>18±1</td>
</tr>
</tbody>
</table>

Values are radioactivity counts divided by the specific radioactivity of the 125I-HDL and are the means±SEM of triplicate determinations given in ng HDL/mg cell protein. Monensin also reduced the amount of radioactivity internalized by control cells by 53% and by cholesterol-treated cells by 73%. The radioactivity released during the chase was 60% and 80% lower for control and cholesterol-treated cells, respectively, in the presence of the ionophore. The composition of the chase media also changed; the proportion of TCA-precipitable material decreased from 46% to 17% for control cells and from 58% to 14% for cholesterol-treated cells (Figure 4).

In the second type of monensin experiment, with the ionophore included during the chase only, the cholesterol-treated cells released 55% more radioactivity than control cells in the presence of monensin and 58% more in its absence (Figure 5). This reflected a 46% increase in internalized radioactivity in cholesterol-treated cells over controls. There was little difference in the amount of radioactivity released in the presence or absence of monensin; the ionophore reduced the release by only 10% for both cell types. There was, however, a major change in the composition of the chase material: monensin caused a decrease in the TCA-precipitable material and an increase in the TCA-soluble radioactivity (Figure 5). For cholesterol-treated cells, the ratio of TCA-precipitable to TCA-soluble radioactivity decreased from 2:1 to 0.24:1 and for control cells it decreased from 0.7:1 to 0.17:1.

Discussion

These results suggest that cells of the human HepG2 hepatoma line can bind, internalize, and resecrete HDL. About 50% of the internalized (trypsin-resistant) radioactivity released by the cells during a 3-hour chase was TCA-precipitable and, therefore, was not lysosomally degraded material. Furthermore, the resecreted material contained the two major HDL apolipoproteins, A-I and A-II, and exhibited the physical characteristics of mature HDL particles.

When cells were not treated with trypsin before chasing, some of the TCA-precipitable material found in the chase medium was likely to be 125I-HDL that had dissociated from the outer surface of the cells. However, with trypsinized cells, dissociation from the surface could not contribute much to the TCA-precipitable material. When cells were washed at 4°C to prevent internalization, 98% of the radioactivity associated with the cells was removed by trypsin, indicating that the protease could effectively remove 125I-HDL associated with the cells if the lipoprotein was accessible on the outer plasma membrane. This correlated very well with the observation that cells trypsinized after being pulsed at 37°C released less than 2% of their radioactivity when the chase incubation was done at 4°C. In contrast, cells pulsed at 37°C, but not trypsinized, released 12% of their label during the 3-hour chase at 4°C.

The release of minimal amounts of radioactivity by trypsinized cells at 4°C demonstrates that internalized 125I-HDL was not passively leaking out of cells due to proteolytic damage to the cellular membrane. The struc-
tural integrity of the cells was, therefore, not compromised by the trypsin treatment. This was confirmed by monitoring the exclusion of trypan blue and the release of lactate dehydrogenase. The trypsinized cells were also viable, as more than 95% were able to adhere to dishes within 5 hours after treatment and proliferate normally.

It is possible that the release of TCA-precipitable radioactivity by trypsinized cells was due to internal damage caused by trypsin, which may have been internalized by the cells during their 10-minute exposure to the protease at 37°C. However, when trypsinized cells were allowed to recover from the effects of the protease and re-attach to the dishes (5 hours), more of the material they released during the subsequent 3-hour chase was precipitable with TCA compared to cells chased for 3 hours immediately after trypsinization. It is thus more likely that internalized trypsin increased the secretion of degradation products rather than that of TCA-precipitable material during the first 3 hours after treatment. The reduction in the release of TCA-precipitable label by untrypsinized cells seen in the presence of monensin or chloroquine also argues against the possibility that the release of intact HDL occurs only in trypsin-treated cells.

The release of labeled HDL particles during the chase is also unlikely to be due to secretion of newly synthesized lipoproteins to which radioactivity could have transferred. HepG2 cells cultured under the conditions used synthesize and secrete mainly disaccharid and some spherical HDL particles, which are much smaller than mature circulating human HDL. The HDL resecreted in the chase medium although they appeared to be modified when they were tested with anti-HDL antiserum. Schmitz et al. described a similar process in cholesterol-laden mouse peritoneal macrophages. Using HDL conjugated to colloidal gold, they presented electron microscopic observations suggesting that the particles released during the chase were endocytosed and recycled to the plasma membrane.

Retroendocytosis is a plausible mechanism for the observed release of internalized labeled HDL during the chase incubation. The process involves the receptor-mediated internalization of a ligand into the endosomal compartment and its subsequent ressecretion from the cell as the endosomes recycle to the plasma membrane. The retroendocytosis of HDL was first postulated to occur in cultured rat aortic smooth muscle cells by Bierman et al. These cells released whole 125I-HDL particles into the chase medium although they appeared to be modified when they were tested with anti-HDL antiserum. Schmitz et al. described a similar process in cholesterol-laden mouse peritoneal macrophages. Using HDL conjugated to colloidal gold, they presented electron microscopic observations suggesting that the particles released during the chase were endocytosed and recycled to the plasma membrane.

### Table 4. Effect of Cholesterol and Monensin on Binding, Internalization, and Release of HDL

<table>
<thead>
<tr>
<th>Cells</th>
<th>Specific binding (4°C)</th>
<th>Internalization (37°C)</th>
<th>Released into chase medium (37°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>48.5</td>
<td>189</td>
<td>74.6</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>74.5</td>
<td>281</td>
<td>115</td>
</tr>
<tr>
<td>Cholesterol+monensin</td>
<td>10.8</td>
<td>82.3</td>
<td>30.1</td>
</tr>
<tr>
<td>Cholesterol+monensin</td>
<td>16.8</td>
<td>75.3</td>
<td>22.9</td>
</tr>
</tbody>
</table>

Values are radioactivity counts divided by the specific radioactivity of 125I-HDL and are the means of duplicate determinations.
evidence suggesting that the conjugates were bound, internalized into endosomes, and subsequently resecreted from the cells. Oram et al. were unable to find evidence of internalization and resecretion by these mouse cells using I-HDL, but Alam et al. found that iodinated HDL appeared to be retroendocytosed by human monocyte-derived macrophages and that the process was up-regulated by cholesterol loading. Recently, Takahashi et al. have also found evidence for internalization and resecretion of I-HDL by rat peritoneal macrophages. They have also clearly shown, using electron microscopy, that HDL conjugated with ferritin or horseradish peroxidase follows the same pathway as transferrin conjugated with horseradish peroxidase in these cells. The pathway entails internalization via coated pits and receptosomes followed by transfer to the trans-Golgi system followed by resecretion via secretory vesicles.

Recently, Tanaka et al. have also presented evidence for a nonlysosomal intracellular pathway for HDL in rat sinusoidal liver cells. These cells internalize I-HDL and secrete TCA-precipitable material during a subsequent chase period; degradation is not a major pathway, as more than 95% of the released material is acid-precipitable. The internalization of HDL was assessed by trypsin treatment (I-HDL) and with fluorescein isothiocyanate-labeled HDL, which showed that the lipoprotein entered an intracellular compartment with an acidic pH, most probably the endosomes.

The observations of Tanaka et al. are consistent with the results we have obtained with monensin in this study. The carboxylate ionophore mediates the transfer of H+ across cell membranes in exchange for Na+, thereby interfering with the acidification of intracellular endosomal and secretory compartments by proton pumps. Consequently, monensin blocks the movement of endosomes which carry recycling molecules such as the receptors for LDL and mannose-glycoproteins. Because the ionophore has no effect on the formation of endosomes and, therefore, on the internalization of plasma membrane proteins, recycling receptors are trapped inside the cell in these vesicles and are unavailable to bind their ligands at the cell surface. The reduction in binding and internalization of I-HDL by cells pre-incubated with monensin is, therefore, consistent with the intracellular entrapment of a recycling receptor-like factor, which is required for the binding and internalization of HDL. Pittman et al. have also observed a 77% decrease in the association of I-apo A-I HDL upon treatment of HepG2 cells with monensin. These monensin effects are also consistent with the recent finding by Takahashi et al. that the retroendocytosis of HDL by macrophages involves the trans-Golgi apparatus and secretory vesicles. Monensin has rapid effects on the trans-Golgi apparatus and secretory vesicles similar to those discussed for endosomes.

Monensin also appeared to effectively inhibit the resecretion of TCA-precipitable material in both untrypsinized and trypsinized cells. This was most clearly seen when trypsinized cells were exposed to the ionophore during the chase period only. The resecretion of internalized HDL was, therefore, dependent on the acidification and recycling of endosomal vesicles or on the movement of secretory vesicles to the surface. In contrast, the delivery of HDL to the lysosome for degradation was not inhibited. In fact, blocking the retroendocytotic pathway caused a compensatory channelling of I-HDL toward the lysosomes for degradation. This markedly changed the composition of the released material with little effect on the total amount of radioactivity released. This effect of monensin was seen whether or not the cells were preincubated with cholesterol, although it was more dramatic with cholesterol-treated cells. In the absence of monensin, the cells exposed to cholesterol released twice as much TCA-precipitable material as they did TCA-soluble radioactivity, but in the presence of the ionophore they released four times more TCA-soluble label than they did TCA-precipitable material.

Although HDL (1.07<d<1.21) contained traces of apo E, the LDL receptor is unlikely to be involved in the retroendocytosis of the lipoprotein. First, in fibroblasts where retroendocytosis of LDL has been documented, retroendocytosed LDL normally represented less than 15% of metabolized LDL, while up to 50% of the metabolized HDL was retroendocytosed lipoprotein in the present study. Second, both monensin and chloroquine markedly increased the amount of LDL retroendocytosed by fibroblasts but inhibited the release of TCA-precipitable HDL radioactivity by HepG2 cells. Third, apo E-poor HDL3 was retroendocytosed by our HepG2 cells to the same extent as total HDL. Fourth and most importantly, cholesterol, which down-regulates the LDL receptor, caused an increase in the amount and relative proportion of retroendocytosed HDL3. The putative apo E or remnant receptor is also unlikely to be involved in the retroendocytosis of HDL because it is minimally regulated by cholesterol.

The expression of a receptor for HDL by HepG2 cells that differs from the LDL receptor was first suggested by the work of Dashti et al. This was supported by Hoeg et al. who demonstrated an increase in the specific association of apo E-free I-HDL when the cells were incubated with cholesterol. Other cell types also demonstrate an increased HDL binding capacity after cholesterol loading. Recently, we have reported the presence of an 80 kDa protein in HepG2 cells, which specifically bound colloidal gold-HDL and was up-regulated by cholesterol. Such findings are in contrast to the well-documented down-regulation of the LDL receptor by cholesterol. In the present study, the cholesterol-induced increase in the specific binding of I-HDL at 4°C was reflected in a proportional increase in both the amount of label internalized and that released during the chase. As in control cells, the increased uptake seen with cholesterol was sensitive to monensin; the ionophore reduced the amount of label internalized to the level observed with control cells in its presence. Evidently, the increased uptake resulted from an increase in the activity of an endosomally recycling receptor-like factor which differs from the LDL receptor.

The cholesterol-induced increase in the release of label during the chase was mainly due to an increase in the release of TCA-precipitable radioactivity; cholesterol had little effect on the release of acid-soluble material.
The increased binding and internalization thus led to an increase in retroendocytosis rather than to an increase in lysosomal degradation. It is, therefore, tempting to suggest that the retroendocytosis of HDL may serve to remove cholesterol from HepG2 cells. Schmitz et al. have suggested such a function for retroendocytosed HDL in cholesteryl-ester-laden macrophages based on the association of gold-labeled HDL with cholesteryl ester storage vesicles in these cells. Also, Alam et al. have recently reported that HDL3 is converted to a larger apo E-containing particle (of HDL2 size and density) during retroendocytosis through cholesteryl-ester-laden macrophages, an observation that is consistent with an enrichment of HDL3 with cholesteryl.

As with macrophages, HDL can cause the efflux of cholesterol from cholesteryl-loaded HepG2 cells; the cholesterol content of such cells has been observed to decrease by 13% during a 6-hour incubation with HDL. These cholesterol-loaded HDL2 cells exhibited increased binding and internalization of HDL even when measured in the presence of heparin to prevent their binding to the LDL receptor via apo E. In other studies with HepG2 cells not loaded with cholesterol, HDL2 had no effect on cellular free or esterified cholesterol content, but it markedly decreased acyl-CoA:cholesterol acyltransferase (ACAT) activity and increased LDL receptor activity, suggesting that the HDL caused a reduction in the regulatory pool of cholesterol without affecting total cellular cholesterol.

The importance of the retroendocytotic pathway for HDL in HepG2 cells can be assessed in the following manner. The results in Table 1 show that equal amounts of TCA-precipitable and TCA-soluble radioactivity are released from internalized (trypsin-resistant) label. Assuming that the same occurs in untrypsinized cells, it can be calculated that degradation, retroendocytosis, and release from the surface membrane (reversible binding) each account for one-third of the released material. In other words, retroendocytosis and reversible binding each account for half of the release of undegraded HDL. Therefore, from the perspective of cellular cholesteryl homeostasis, retroendocytosis can potentially move as much cholesteryl as reversible binding.

In the present study, the amount of HDL retroendocytosed was too small to allow reliable compositional analysis of lipids, but we found that the apo A-I content of the retroendocytosed HDL was lower than that of the original HDL. Also, both the internalized and resecreted HDL appeared to contain proportionally less of the larger HDL2 type particles compared to HDL not exposed to the cells. These differences may represent true changes to some of the particles as they journeyed through the cells or they may result from preferential uptake and/or resecretion of particles with the observed characteristics. As these experiments were done with HepG2 cells grown under normal culture conditions, it remains to be seen whether retroendocytosed HDL participates in the efflux of cholesterol from cholesteryl-treated HepG2 cells with observable changes in particle characteristics.

In conclusion, our results indicate that HepG2 cells, which can bind, internalize, and degrade HDL, are also able to resecrete the lipoprotein by the process of retroendocytosis. Furthermore, the results with cholesteryl and monensin suggest that the binding, internalization, and subsequent degradation or resecretion of HDL involves the recycling of a regulated receptor-like molecule between the outer plasma membrane and the acidic endosomal compartment of the cell.

Acknowledgments
We thank Anne Jenkins for her expert assistance and Kath Illes for preparation of the manuscript. We are also indebted to Mavis Abbey and Peter Clifton for enlightening discussions.

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doi: 10.1161/01.ATV.10.4.582
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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