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

Ambrosios M. Kambouris, Paul D. Roach, G. Dennis Calvert, and Paul J. Nestel

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 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 60%, and increased the release of acid-soluble material by 93%. 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)

**H**igh 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 chylomicrons 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, 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 cholesterol 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.

---

From CSIRO, Division of Human Nutrition, Kintore Avenue, Adelaide, and the Department of Biochemistry and Chemical Pathology, School of Medicine, The Flinders University of South Australia, Bedford Park, SA, Australia.

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.

Address for correspondence: Paul D. Roach, CSIRO, Division of Human Nutrition, P.O. Box 10041 Gouger Street, Adelaide, SA 5000, Australia.

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) ethylenediaminetetraacetate (EDTA) were from Flow Laboratories (Irvine, UK). Culture flasks were from Nunc (Roskilde, Denmark). Bovine serum albumin (BSA), chloroquine, chloramphenicol, cholesterol (99%+), genticyn, and monensin were from Sigma Chemical Company (St. Louis, MO). Sodium 125I-iodine 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 (1.25<d<1.21) were prepared by sequential preparative ultracentrifugation23 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 gentamycin (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 lecinthin:cholesterol acyl transferase (LCAT) activity.3 Protein was determined by the method of Lowry et al.24

Radiiodination of High Density Lipoprotein

HDL and HDL3 were radiiodinated by the iodine monochloride technique25 as modified for lipoproteins26 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),27 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.13 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 radioactivity28; 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 Ti 50.3 Beckman rotor. The radioactive material that floated was subjected to electrophoresis on 2.5% to 27% concave gradient polyacrylamide gels29 (Gradient Labs Pty, Sydney, Australia). The gels were autoradiographed on Kodak film at −70°C, 30 and the autoradiographs were scanned with an LKB 2202 Ultrascan laser densitometer interfaced with a Hewlett-Packard computing integrator.

To characterize the radioactivity released by the cells, the chase medium was adjusted to d = 1.21 g/ml for ultracentrifugation. Electrophoresis of the floating material followed by autoradiography and densitometric analysis were done as described above for the internalized radioactivity. The apolipoprotein composition of the TCA-precipitable radioactive material of the chase medium was analyzed by electrophoresis on a 10% polyacrylamide gel containing SDS27 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 treatment, 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 trypsin 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>Released into chase medium</th>
<th>TCA-precipitable</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell-associated before chase</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>220±15</td>
<td>26±3</td>
</tr>
<tr>
<td>Chased at 4°C</td>
<td>90±4</td>
<td>1.5±0.3</td>
</tr>
<tr>
<td>Chased at 37°C</td>
<td>220±15</td>
<td>105±9</td>
</tr>
<tr>
<td>Trypsin treated</td>
<td>90±4</td>
<td>29±1</td>
</tr>
</tbody>
</table>

Values are given in ng HDL/mg cell protein and are radioactivity counts divided by the specific radioactivity of \(^{125}\text{I}-\text{HDL.}\) They are the mean±SEM of triplicate determinations.

HepG2 cells were incubated with 100 \(\mu\text{g/ml}\) \(^{125}\text{I}-\text{HDL}\) 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 either 4°C or 37°C. HDL=high density lipoprotein, EDTA=ethylenediaminetetraacetate, TCA=trichloroacetic acid.

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. Gradients of both the internalized and resecreted labeled moieties were similar to that of marker \(^{125}\text{I}-\text{HDL}\) (Figure 2).

Chloroquine had totally different effects. The lysosomal inhibitor did not inhibit the association of \(^{125}\text{I}-\text{HDL}\) 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 \(^{125}\text{I}-\text{HDL}\) 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 \(^{125}\text{I}-\text{HDL}\) and the release of internal-
ized radioactivity during the chase were also increased by 50%. Analysis of the chase media revealed that cholesterol caused a 92% increase in TCA-precipitable chase material, but only a 21% increase in TCA-soluble material. Exposing the cells to monensin for 2 hours before pulsing with radiolabeled HDL resulted in a 77% decrease in specific binding at 4°C whether or not the cells had been exposed to cholesterol (Table 4). 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 $^{125}$I-HDL that had dissociated from the outer surface of the cells. However, with trypsinated cells, dissociation from the surface could not contribute much to the TCA-precipitable material. When cells were pulsed 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 $^{125}$I-HDL associated with the cells if the lipoprotein was accessible on the outer plasma membrane. This correlated very well with the observation that cells trypsinated 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 trypsinated, released 12% of their label during the 3-hour chase at 4°C.

The release of minimal amounts of radioactivity by trypsinated cells at 4°C demonstrates that internalized $^{125}$I-HDL was not passively leaking out of cells due to proteolytic damage to the cellular membrane. The struc-
The retroendocytosis of HDL was first postulated to occur by Bierman et al. These cells released whole \( ^{125}\text{I-HDL} \) particles into the chase medium although they appeared to be modified in cultured rat aortic smooth muscle cells by Bierman et al. The HDL resecreted in the chase medium is identical HDL particles, which are much smaller than mature sized lipoproteins to which radioactivity could have transferred. HepG2 cells cultured under the conditions used to synthesize and secrete mainly discoidal and some spherical HDL particles, which are much smaller than mature circulating human HDL. The HDL resecreted in the present experiments exhibited a particle size similar to that of the mature human HDL used during the pulse.

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 resecretion 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 \( ^{125}\text{I-HDL} \) particles into the chase medium although they appeared to be modified when they were tested with anti-HDL antisemur. Schmitz et al. described a similar process in cholesterol-laden mouse peritoneal macrophages. Using HDL conjugated to colloidal gold, they presented electron microscopic

---

### 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>Control + 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 \( ^{125}\text{I-HDL} \) 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 125I-HDL, but Alam et al. found that iodinated HDL appeared to be retroendocytosed by human monocyte-derived macrophages and that the process was upregulated by cholesterol loading. Recently, Takahashi et al. have also found evidence for internalization and resecretion of 125I-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 125I-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-soluble. The internalization of HDL was assessed by trypsin treatment (125I-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 carboxylic 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 125I-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 125I-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 125I-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 125I-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-HDL3 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 125I-HDL3 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. Schmidt 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 HDL₄ size and density) during retroendocytosis through cholesteryl-laden macrophages, an observation that is consistent with an enrichment of HDL₃ with cholesterol.

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 15% during a 6-hour incubation with HDL. These cholesteryl-loaded HepG2 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, HDL₃ 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 cholesterol homeostasis, retroendocytosis can potentially move as much cholesterol 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 resorbed HDL appeared to contain proportionally less of the larger HDL₃ 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 resorption 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 cholesterol-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 cholesterol 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.

References

10. Arbeeny CM, Rifflc VA, Eder HA. The uptake of the apoprotein and cholesteryl ester of high density lipoproteins by perfused rat liver. Biochim Biophys Acta 1987;917:9-17
20. Slotte JP, Oram JF, Blerman EL. Binding of high density lipoproteins to cell receptors promotes translocation of cholesterol from intracellular membranes to the cell surface. J Biol Chem 1987;262:12904–12907

Index Terms: retroendocytosis • high density lipoproteins • HepG2 cells • monensin • chloroquine • trypsin • cholesterol
Retroendocytosis of high density lipoproteins by the human hepatoma cell line, HepG2.
A M Kambouris, P D Roach, G D Calvert and P J Nestel

doi: 10.1161/01.ATV.10.4.582

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/10/4/582