Lipid metabolism in cultured cells. XVIII. Comparative uptake of low density and high density lipoproteins by normal, hypercholesterolemic and tumor virus-transformed human fibroblasts

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Abstract Serum lipoproteins control cell cholesterol content by regulating its uptake, biosynthesis, and excretion. Monolayers of cultured fibroblasts were used to study interactions with human high density (HDL) and low density (LDL) lipoproteins doubly labeled with [^{3}H]cholesterol and ^{125}I in the apoprotein moiety. In the binding assay for LDL, the absence of specific LDL receptors in type II hypercholesterolemic fibroblasts was confirmed, whereas monolayers of virus-transformed human lung fibroblasts (VA-4) exhibited LDL binding characteristics essentially the same as normal lung fibroblasts. In the studies of HDL binding, specific HDL binding sites were demonstrated in normal and virus-transformed fibroblasts. In addition, type II hypercholesterolemic cells, despite the loss of LDL receptors, retained normal HDL binding sites. No significant competition was displayed between the two lipoprotein classes for their respective binding sites over a 5-fold concentration range. In VA-4 cells, the amount of lipoprotein required to saturate half the receptor sites was 3.5 μg/ml (9×10^{-9} M) for LDL and 9.1 μg/ml (9×10^{-8} M) for HDL. Pronase treatment reduced LDL binding by more than half but had no effect on HDL binding. Chloroquine, a lysomal enzyme inhibitor, stimulated net LDL uptake 3.5-fold by increasing internalized LDL but had essentially no effect on HDL uptake. Further experiments were conducted using doubly labeled lipoproteins to characterize the interaction of LDL and HDL with cells. While the cholesterol and protein moieties of LDL were incorporated into cells at similar rates, the uptake of the cholesterol moiety of HDL was 5 to 10 times more rapid than that of the protein component. Furthermore, the apoprotein component of LDL is extensively degraded following exposure, whereas the apoprotein moiety of HDL retains its macromolecular chromatographic characteristics. These results indicate that HDL and LDL bind to cultured cells at separate sites and that further processing of the two lipoprotein classes appears to take place by fundamentally different mechanisms.—**Wu, J-D., J. Butler, and J. M. Bailey.** Lipid metabolism in cultured cells XVIII. Comparative uptake of low density and high density lipoproteins by normal, hypercholesterolemic, and tumor virus-transformed human fibroblasts. *J. Lipid Res.* 1979. **20:** 472–480.

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Cell cholesterol content represents the balance of three processes: cholesterol transport into the cell, cholesterol excretion from the cell, and de novo cholesterol synthesis. All of these processes are directly or indirectly controlled by serum lipoproteins. The first indication that cultured cells possess a regulatory system for cholesterol metabolism came from studies on cells grown in chemically defined media (1). It was found that whereas cultured cells grown in serum take up cholesterol and exhibit a low rate of cholesterol synthesis, in synthetic lipid-free medium the cholesterol requirements were entirely met by de novo biosynthesis. In 1973, Brown and his colleagues (2–4) demonstrated the presence of a specific receptor on the surface of cultured human fibroblasts which binds plasma low density lipoproteins. The membrane-bound LDL receptors control cholesterol metabolism in extrahepatic tissues through the regulation of HMG CoA reductase (5–9). While the function of LDL in cholesterol regulation has been elucidated, the role of HDL in lipid metabolism is only partially understood at the present time. The excretion of cholesterol from cultured cells into the medium was shown to be related to the serum concentration in the medium (10). It was shown that most of the activity was contributed by the serum α-globulins (11). Many studies (12–15) have indicated that HDL promotes the removal of cholesterol from peripheral tissues and may protect against the development of atherosclerosis. It has also been proposed that HDL may function as a source of apoC and apoE for interaction with triglyceride-rich VLDL and chylomicrons, and also as a cosubstrate for lecithin–cholesterol acyl transferase and cholesterol esterification.

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The confirmation of LDL receptors and their regulatory role in cholesterol uptake and biosynthesis leads to the belief that other receptor sites are associated with proposed HDL functions. The aims of this study were to compare the specific binding of HDL and LDL in both normal and abnormal human fibroblasts and to characterize the mechanism of uptake of the two lipoproteins in the various cell types examined.

MATERIALS AND METHODS

Chloroquine (Winthrop Laboratories), cycloheximide (Gibco, Grand Island, NY), sodium heparin (Upjohn Co.) and pronase (Calbiochem, San Diego), were used as purchased without purification. $Na^{125}I$ and labeled cholesterol (1,2-^{3}H (N)) were purchased from New England Nuclear Corp. (Boston, MA). Cholesterol was purified by thin-layer chromatography before use.

Normal human fibroblasts (Bo Mat and T.J.) and normal human fetal lung fibroblasts (WI-26 and WI-38) were from the Wistar Institute and American Type Cell Collection, respectively. VA-4 cells (SV-40 viral-transformed product of WI-26) were a gift from the Wistar Institute. L-2071 cells (mouse transformed fibroblasts) were obtained from Dr. Virginia Evans, N.I.H. Homozygous familial hypercholesterolemic (FH) skin fibroblasts (GM-361) and heterozygous familial hypercholesterolemic skin fibroblasts (GM-483) were obtained from The Institute for Medical Research, NJ.

Cell cultures (except L-2071 cells) were grown in a CO_2 incubator at 37°C in Eagle's minimum essential medium (Grand Island Biological Co., Grand Island, NY) supplemented with $NaHCO_3$ (2.2 g/l), 10% fetal bovine serum, 1% essential vitamin mixture (100×), and antibiotics (50 units/ml of penicillin and 50 μg/ml of streptomycin). L-2071 cells were cultured in a chemically defined medium, NCTC-135. This medium was prepared by modification (16) of the method of Evans et al. (17). All lipid sources were omitted and lipid-soluble vitamins were dissolved in ethanol. Plastic 75-cm² T flasks containing normal and transformed fibroblasts took 5–6 days and 3–4 days, respectively, to reach confluency as determined by microscopic examination. Flasks of confluent cells were then treated with 0.25% trypsin and seeded into four 25-cm² T flasks. The trypsinization time was found to be 15 and 4 min for normal and transformed cells, respectively. Cells were grown an additional 3 days to reach confluency. The protein content of normal and transformed cells was found to be 160–200 μg and 550–750 μg per flask, respectively, by the method of Lowry et al. (18).

Preparation of radioactively labeled lipoproteins

Human very low density lipoprotein (VLDL) (d 0.95–1.006 g/ml), low density lipoprotein (d 1.019–1.063 g/ml), high density lipoprotein (d 1.063–1.21 g/ml), and lipoprotein-free serum (LPFS) (d > 1.21 g/ml), were isolated according to standard ultracentrifugation flotation techniques using solid KBr for flotation adjustment (19). The purity of lipoprotein fractions was confirmed by polyacrylamide gel electrophoresis, agarose gel electrophoresis, and immunoelectrophoresis.

The LDL fraction was concentrated and iodinated by a modification of the iodine monochloride method of McFarlane (20). A solution of LDL (0.4 ml, 16 mg of protein) was diluted with 1 M glycine buffer (0.75 ml, pH 10) at 0°C. Stock $Na^{125}I$ solution (10 mCi in 0.1 ml of 0.05 N NaOH diluted to 0.5 ml with glycine buffer) and freshly prepared iodine monochloride stock solution (15 μl, 4.2 mg/ml) were mixed with LDL and incubated for 5 min in an ice bath. The reactants were diluted with buffer containing 20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, and 0.3 mM EDTA (buffer A), filtered through a PM-10 Diaflo membrane, and washed repeatedly with buffer A to remove unbound ^{125}I. The final preparation was sterilized by filtration through a 0.45-μm Millipore filter using a Swinnex syringe adapter. The HDL fraction was iodinated by the ICl method of McFarlane (20) modified by incubating the mixture for 1 hr in an ice bath instead of 5 min.

For the preparation of doubly labeled lipoproteins, [^{3}H]cholesterol in ethanol was dried in a thin film on the walls of a capped test tube placed on a roller drum turning at 9 rph. The ^{125}I-labeled lipoprotein was then added, and the uptake of [^{3}H]cholesterol into the lipoprotein was monitored as described previously (21). This process was performed using sterile conditions and the doubly labeled lipoprotein was used without further treatment.

Radioactive samples containing no water were counted in a toluene-based scintillation fluid containing the phosphors 2,5-diphenyloxazole (PPO), 4 g/l, and P-bis(2-(phenyloxazolyl))-benzene (POPOP), 50 mg/l (Yorktown Research). Samples in aqueous solution were counted in the premixed scintillant Hydromix (Yorktown Research). Prepared counting vials were counted in a Beckman LS-250 scintillation counter. Quench correction curves were constructed for each isotope using external standard quench ratios.

^{125}I-labeled LDL and ^{125}I-labeled HDL binding assay

The interaction of ^{125}I-labeled LDL or ^{125}I-labeled HDL with cell monolayers was determined according

TABLE 1. Binding[a] of ^{125}I-labeled LDL to normal, hypercholesterolemic, and tumor virus-transformed human fibroblasts

Cell Type	^{125}I-Labeled LDL Bound		
	Total	Nonspecific	Specific
	ng LDL protein/mg cell protein		
Normal			
T.J. skin fibroblast	181 ± 6	61 ± 6	120 ± 12
WI-38 lung fibroblast	158 ± 19	23 ± 1	135 ± 20
Tumor			
VA-4 transformed lung fibroblast	207 ± 19	55 ± 2	152 ± 21
Hypercholesterolemic			
GM-361 homozygous type II skin fibroblast	16 ± 2	8 ± 1	8 ± 3
GM-483 heterozygous type II skin fibroblast	99 ± 37	56 ± 1	43 ± 38

Cells were grown for 4 days until confluent in Eagles's minimum essential medium supplemented with 10% fetal bovine serum and for a further 24 hr in medium supplemented with lipoprotein-free serum. Total binding was measured in cultures supplemented with ^{125}I-labeled LDL at a level of 5 μg protein/ml, and nonspecific binding was measured in similar cultures to which a 100-fold excess of unlabeled LDL was added as described by Goldstein and Brown (24). After 3 hr cultures were washed, the radioactivity in lipids was extracted, and the protein-bound activity remaining was determined as described in Methods.

[a] Unless otherwise specified, the term "binding" refers to total uptake of ^{125}I-labeled protein and will include internalized materials.

to a modification of the methods of Brown and Goldstein (22, 23). Confluent cultures of T.J., Bo Mat, WI-38, GM-361, GM-483, and VA-4 cells were incubated for 24 hr in Eagle's minimum essential medium (serum-free) supplemented with lipoprotein-free serum (2.5 mg protein/ml). The following day, the medium was removed and replaced with 2 ml of medium that contained 5 mg of lipoprotein-free serum and 10 μg of ^{125}I-labeled LDL or ^{125}I-labeled HDL. Cultures were incubated in triplicate; excess unlabeled LDL or HDL (1 mg) was added to one control culture of each set to determine the nonspecific binding of each lipoprotein. After 3 hr of incubation at 37°C, the cell monolayer was washed twice with 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, and bovine serum albumin (2 mg/ml), and then washed seven times with the same buffer without albumin. In the LDL binding assay, the washed cell monolayers were extracted with ether–ethanol 1:1 to remove the ^{125}I associated with lipid and the cell residue was digested in 0.5 N NaOH (2.0 ml) overnight. In the case of the HDL binding assay, where ^{125}I in the lipid moiety was negligible, the washed cells were digested in 0.5 N NaOH overnight. Aliquots were used for

TABLE 2. Binding of ^{125}I-labeled HDL to normal, hypercholesterolemic, and tumor virus-transformed human fibroblasts

Cell Type	^{125}I-Labeled HDL Bound		
	Total	Nonspecific	Specific
	ng HDL protein/mg cell protein		
Normal			
BA DEL human skin fibroblast	142 ± 25	25 ± 3	117 ± 2
WI-38 human lung fibroblast	150	50	100
Tumor			
WI-26-VA-4 transformed lung fibroblast	154	46	108
Hypercholesterolemic			
GM-361 homozygous type II skin fibroblast	137 ± 1	26 ± 1	111 ± 2
GM-483 heterozygous type II fibroblast	206 ± 6	28 ± 2	178 ± 7

Cells were grown until confluent in Eagle's minimum essential medium supplemented with 10% fetal bovine serum and for an additional 24 hr in medium supplemented with lipoprotein-free serum. Total binding was measured in cultures supplemented with ^{125}I-labeled HDL protein at a level of 5 μg/ml and nonspecific binding was measured in similar cultures to which a 100-fold excess of unlabeled HDL was added. After 3 hr, cultures were washed and radioassayed as described in Methods.

protein determination and scintillation counting. The specific binding of each cell line was determined from the difference between total binding and that in control cultures and expressed as ng of LDL or HDL protein bound per mg cell protein.

^{125}I-labeled LDL or ^{125}I-labeled HDL binding to VA-4 cells

The binding of ^{125}I-labeled LDL or ^{125}I-labeled HDL to VA-4 cells was determined at several lipoprotein concentrations ranging from 0 to 40 μg protein/ml medium. The data were expressed as ^{125}I-labeled lipoprotein bound/free ^{125}I-labeled lipoprotein in 2 ml of medium versus ^{125}I-labeled lipoprotein bound. K_d (concentration of ^{125}I-labeled lipoprotein to achieve one-half saturation of cell surface receptor sites) and V_{max} were determined by the slope and interception on the y-axis, respectively.

Specificity of ^{125}I-labeled LDL or ^{125}I-labeled HDL binding to VA-4 cells

Interaction of VA-4 monolayers with ^{125}I-labeled LDL was studied at 5 μg/ml of ^{125}I-labeled LDL in the presence and absence of native LDL or native HDL. In a similar experiment, the interaction of ^{125}I-labeled HDL with VA-4 cell monolayers was studied at 10 μg/ml of ^{125}I-labeled HDL. Total binding of ^{125}I-labeled HDL to VA-4 cells was also determined at 10 μg/ml of ^{125}I-labeled HDL protein in the presence of excess native HDL and LDL. The competition between LDL and HDL for both specific HDL and LDL binding sites was investigated.

Influence of chloroquine and cycloheximide on specific uptake of ^{125}I-labeled LDL or ^{125}I-labeled HDL by VA-4 cells

The effect of drugs on the interaction of ^{125}I-labeled LDL or ^{125}I-labeled HDL to VA-4 cells was investigated. In these studies, the standard binding assay was conducted in the presence of 100 μM chloroquine, 20 μg of cycloheximide, or the combination of 100 μM chloroquine and 20 μg of cycloheximide. The total lipoprotein binding was determined after 10 hr at 37°C in the presence of each drug.

The interaction of ^{125}I-labeled LDL with VA-4 cells was studied in the presence and absence of 100 μM chloroquine. After 10 hr of incubation, cell monolayers were washed as in the standard procedure. These cells were then suspended in 2.5 ml of 5 mg/ml heparin solution (5,000 units/ml) at room temperature for 1 hr. The mixture was centrifuged and the cell pellet was washed once with 2 ml of Tris-HCl buffer. The supernatant and washed media were combined and counted. Cell pellets were digested in 0.5 N NaOH overnight. Aliquots of cell digest were taken for protein determination and for liquid scintillation counting. The radioactive iodine in the supernatant represented the surface-bound ^{125}I-labeled LDL, while the radioactivity remaining in the cell pellet was used to quantitate the internalized ^{125}I-labeled LDL.

TABLE 3. Specificity of ^{125}I-labeled LDL binding to SV-40 virus-transformed human fibroblasts (WI-26-VA-4)

Medium	Total LDL Binding
	ng LDL protein bound/ mg cell protein
5 μg ^{125}I-labeled LDL protein/ml	208 ± 20
5 μg ^{125}I-labeled LDL protein/ml and 100-fold excess native LDL	55 ± 2
5 μg ^{125}I-labeled LDL protein/ml and 5-fold excess native LDL	137 ± 5
5 μg ^{125}I-labeled LDL protein/ml and 5-fold excess native HDL	202 ± 11

The interaction of VA-4 cells with ^{125}I-labeled LDL was determined at 5 μg ^{125}I-labeled LDL protein/ml in the presence of excess native LDL and HDL. The competition of HDL and LDL for the specific LDL receptor sites was investigated.

Influence of pronase on the uptake of ^{125}I-labeled LDL or ^{125}I-labeled HDL by VA-4 cells

VA-4 cell monolayers were preincubated with 2 ml of Eagle's minimum essential medium in the presence and absence of 6 μg of pronase. After 25 min of incubation, the cells were washed twice with medium and then incubated in a solution of 10 μg/ml of ^{125}I-labeled LDL or ^{125}I-labeled HDL for 3 hr. The total uptake of ^{125}I-labeled lipoprotein by VA-4 cells was measured.

Uptake of free ^{3}H-labeled cholesterol and ^{125}I-labeled apolipoprotein by L-2071 cells

L-2071 cells previously grown on NCTC-135 medium without serum were incubated with doubly labeled LDL or HDL. At timed intervals, cells were harvested and the presence of [^{3}H]cholesterol and ^{125}I-labeled apolipoprotein in the lipid extract was assessed by dual isotope liquid scintillation counting. The cell residue was analyzed for protein content. The ratio ^{3}H/^{125}I found in the cells after harvesting was normalized by dividing each ratio by the ^{3}H/^{125}I ratio of the original lipoprotein. The medium remaining after the HDL incubation was chromatographed on a Sephadex G-200 column to determine the extent to which the ^{125}I label was still associated with the protein.

RESULTS

Quantitative comparisons of specific LDL or HDL binding were made after 3 hr of incubation at 37°C.

TABLE 4. Specificity of ^{125}I-labeled HDL binding to SV-40 virus-transformed human lung fibroblasts (WI-26-VA-4)

Medium	Total HDL Binding
	ng HDL protein bound/ mg cell protein
10 μg ^{125}I-labeled HDL protein/ml	255 ± 47
10 μg ^{125}I-labeled HDL protein/ml and 5-fold excess native HDL	104
10 μg ^{125}I-labeled HDL protein/ml and 25-fold excess native HDL	54 ± 1
10 μg ^{125}I-labeled HDL protein/ml and 100-fold excess native HDL	58 ± 3
10 μg ^{125}I-labeled HDL protein/ml and 5-fold excess native LDL	226 ± 6
10 μg ^{125}I-labeled HDL protein/ml and 25-fold excess native LDL	123 ± 1
10 μg ^{125}I-labeled HDL protein/ml and 100-fold excess native LDL	83

Interaction of HDL with VA-4 cells was studied at 10 μg/ml of ^{125}I-labeled HDL in the presence of excess native LDL and HDL. The competition of LDL with HDL for the specific HDL receptors was determined.

The correction for nonspecific binding was measured by the radioactivity bound to the monolayers in the presence of a 100-fold excess of unlabeled lipoprotein (**Table 1**). In the case of the LDL binding assay, normal skin fibroblasts displayed specific binding with an average of 120 ng of ^{125}I-labeled LDL protein per mg of cell protein. While heterozygous type II skin fibroblasts bound 43 ng of ^{125}I-labeled LDL protein per mg of cell protein, homozygous type II skin fibroblasts showed essentially no binding activity, averaging less than 8 ng of ^{125}I-labeled LDL protein bound per mg protein. Normal human lung fibroblasts behaved essentially the same as skin fibroblasts, averaging 135 ng of LDL protein bound per mg of protein. VA-4 tumor cells grown to the monolayer stage bound 152 ng/mg, which was not significantly different than the binding of the normal diploid cells.

The results of the HDL binding assay (**Table 2**) show that normal skin and lung fibroblasts bind 117 and 110 ng HDL protein/mg cell protein, respectively. Monolayers of VA-4 cells bind 108 ng of ^{125}I-labeled HDL protein per mg of cell protein. In contrast to the LDL binding, homozygous and heterozygous type II hypercholesterolemic cells bound 111 and 178 ng HDL protein per mg of cell protein, respectively.

Studies on the concentration dependence of binding indicated that half saturation was 3.5 μg/ml (9×10^{-9} M) for LDL and 9.1 μg/ml (9×10^{-8} M) for HDL. The maximum amount of lipoprotein bound at specific sites was 0.36 and 0.48 μg/mg cell protein for LDL and HDL, respectively.

The specificity of ^{125}I-labeled LDL binding to VA-4 cells was investigated (**Table 3**). The results showed that a 5-fold excess of native LDL decreased ^{125}I-labeled LDL binding by 34%. However, a 5-fold excess of native HDL had no effect on the ^{125}I-labeled LDL binding. In similar experiments using HDL, the results (**Table 4**) showed that a 5-fold excess of native HDL reduced HDL binding by more than 60%. However, a 5-fold excess of native LDL had almost no effect on the HDL binding. A 25-fold excess of native HDL eliminated most of the specific HDL binding while the same quantity of native LDL only displaced 50% of the bound HDL.

The effect of chloroquine on specific uptake of ^{125}I-labeled LDL or ^{125}I-labeled HDL by VA-4 cells was investigated. The data (**Table 5**) showed that 100 μM chloroquine stimulated ^{125}I-labeled LDL uptake 3.5-fold. In the presence of both chloroquine and cycloheximide, the chloroquine effect was abolished while cycloheximide alone decreased LDL binding, a result consistent with its known effects on receptor synthesis.

When ^{125}I-labeled HDL binding was studied in the presence of chloroquine and cycloheximide, it was observed that chloroquine had essentially no effect on the HDL binding, whereas cycloheximide decreased HDL binding by 30% (**Table 6**).

The chloroquine enhancement of ^{125}I-labeled LDL uptake by VA-4 cells was further investigated in the presence of heparin. The results (**Table 7**) indicated that most of the stimulation in binding was due to the internalization (heparin-nonreleaseable ^{125}I) of ^{125}I-labeled LDL. Chloroquine had essentially no effect on the membrane-bound (heparin-releaseable) ^{125}I-labeled LDL.

The influence of pronase on the membrane-bound LDL or HDL receptors was studied. The data (**Table 8**) showed that pronase destroyed more than half of the LDL binding; however, the same enzyme had essentially no effect on HDL binding.

TABLE 5. Influence of chloroquine and cycloheximide on specific uptake of ^{125}I-labeled LDL by VA-4 cells

Medium	Specific LDL Binding
	ng LDL protein bound/ mg cell protein
5 μg ^{125}I-labeled LDL protein/ml	162 ± 15
5 μg ^{125}I-labeled LDL protein/ml and 100 μM chloroquine	523 ± 44
5 μg ^{125}I-labeled LDL protein/ml and 100 μM chloroquine and 10 μg/ml cycloheximide	165 ± 15
5 μg ^{125}I-labeled LDL protein/ml and 10 μg/ml cycloheximide	94 ± 8

VA-4 cell monolayers were studied for their ability to bind ^{125}I-labeled LDL in the presence of 100 μM chloroquine and 20 μg cycloheximide, alone and in combination for 10 hr at 37°C.

Studies using doubly labeled lipoproteins gave qualitatively different patterns for HDL and LDL (**Table 9**). Following an initial binding phase during which the normalized [^{3}H]cholesterol to ^{125}I-labeled apoprotein ratio was 1:1 for both lipoproteins, there was a progressive increase in the total amount of radioactivity taken up during the next few hours for both lipoproteins (**Fig. 1**). The normalized ratio of ^{3}H/^{125}I taken up for LDL did not show striking changes, whereas for HDL there was a progressive increase in these ratios, indicating the preferential transfer of the cholesterol moiety. The medium remaining after the HDL incubations was chromatographed on a Sephadex G 200 column (**Fig. 2**). The results indicated that the bulk of the ^{125}I-labeled protein that remained in the medium had not undergone significant breakdown or modification consequent to removal of the cholesterol.

DISCUSSION

The present study demonstrated the existence of both LDL and HDL receptors in VA-4 cells. The saturation plots further indicated the high affinity of these binding sites (association constants are 9×10^9 M and 9×10^8 M for LDL and HDL, respectively). The specificity of LDL binding to VA-4 cells was supported by the fact that native LDL competed with ^{125}I-labeled LDL for binding and that native HDL showed no ability to displace LDL. The specificity of HDL binding sites and their very weak cross reaction with LDL were also shown by these experiments.

The nature of these lipoprotein receptors was investigated in the presence of chloroquine (a lysosomal enzyme inhibitor), cycloheximide (an inhibitor of protein synthesis), and pronase (a proteolytic enzyme).

TABLE 6. Influence of chloroquine and cycloheximide on uptake of ^{125}I-labeled HDL by VA-4 cells

Medium	Total HDL Binding
	ng HDL protein bound/mg cell protein
10 μg ^{125}I-labeled HDL protein/ml	255 ± 47
10 μg ^{125}I-labeled HDL protein/ml and 100-fold excess native HDL	55 ± 1
10 μg ^{125}I-labeled HDL protein/ml and 100 μM chloroquine	227 ± 13
10 μg ^{125}I-labeled HDL protein/ml and 100 μM chloroquine and 10 μg/ml cycloheximide	199 ± 19
10 μg ^{125}I-labeled HDL protein/ml and 10 μg/ml cycloheximide	185 ± 14

The uptake of ^{125}I-labeled HDL by VA-4 cells was studied in the presence of 100 μM chloroquine and 20 μg cycloheximide alone and in the presence of drugs was determined after 10 hr at 37°C.

TABLE 7. Localization of chloroquine effect on uptake of ^{125}I-labeled LDL by VA-4 cells

Medium	Cell Surface Bound LDL	Internalized LDL
	ng LDL protein/mg cell protein	
10 μg ^{125}I-labeled LDL protein/ml	208 ± 5	181 ± 8
10 μg ^{125}I-labeled LDL protein/ml and 100 μM chloroquine	166 ± 24	449 ± 46

The interaction of ^{125}I-labeled LDL with VA-4 cells was studied at 10 μg/ml of ^{125}I-labeled protein in the presence and absence of 100 μM chloroquine. After 10 hr of incubation, cells were washed and incubated for 1 hr with 2.5 ml of 5 mg/ml heparin solution at room temperature. The mixture was centrifuged, and aliquots of both supernatant and residue were counted. The radioactivity in the supernatant represented surface-bound ^{125}I-labeled LDL, while the radioactivity remaining in the pellet was used to quantitate the internalized ^{125}I-labeled LDL.

The results indicated that chloroquine stimulated ^{125}I-labeled LDL uptake 3.5-fold but had essentially no effect on the HDL uptake. Cycloheximide decreased both LDL and HDL binding, presumably by the inhibition of the synthesis of the membrane-binding proteins. Furthermore, pronase-treated VA-4 cells exhibited normal uptake of ^{125}I-labeled HDL despite the fact that similar treatment destroyed the LDL binding. The sensitivity of LDL binding to pronase digestion and cycloheximide treatment strongly suggested that a membrane protein is an essential element in this binding. Similarly, the decrease of HDL binding by cycloheximide probably indicates that proteins are involved in HDL binding also. However, the insensitivity of HDL binding to proteolytic treatment implies that HDL binding sites are distinct from the high-affinity LDL binding sites and also that these HDL binding sites are either intrinsically resistant to pronase digestion or are incorporated in the membrane in a manner that renders them less accessible to the enzyme.

The mechanisms of uptake of both LDL and HDL by cultured cells is further contrasted when doubly

TABLE 8. Influence of pronase on uptake of ^{125}I-labeled LDL or HDL by VA-4 cells

Pretreatment of Cells	Total Binding	
	LDL	HDL
	ng lipoprotein bound/mg cell protein	
Control	512 ± 67	221 ± 1
Pronase	230 ± 72	271 ± 31

VA-4 cell monolayers were incubated with 2 ml of Eagle's minimum essential medium with and without 6 μg of pronase. After 25 min of incubation with pronase, the cells were washed and then incubated an additional 3 hr with 10 μg/ml of ^{125}I-labeled LDL or HDL protein. Total binding of ^{125}I-labeled LDL and HDL to VA-4 cells was measured.

TABLE 9. Uptake of [^{3}H]cholesterol and ^{125}I-labeled human HDL by L-2071 cells grown on NCTC-135 medium

Experiment	Time	HDL Added	Cell Protein	Total ^{3}H	Total ^{125}I	Radioactivity Uptake as % of Total cpm/mg Cell Protein		Ratio ^{3}H/^{125}I	Normalized Ratio ^{3}H/^{125}I
						[^{3}H]Cholesterol	[^{125}I]HDL		
		μg/bottle	*mg/bottle*	*cpm*	*cpm*				
I	0	167		22,530	5,590			4.0	1.0
	24 hr		0.706	8,685	145	54.8	3.68	60.0	15.0
II	0	16		21,840	6,800			3.2	1.0
	1 hr		0.57	1,886	70	15.1	1.76	26.9	8.4
	3 hr		0.92	4,540	116	22.5	1.84	39.1	12.2
	6 hr		1.53	6,760	176	20.2	1.70	38.4	12.0
III	0	16		23,220	4,380			5.3	1.0
	10 min		0.326	306	30	3.98	2.15	10.4	1.97
	30 min		0.600	802	59	4.83	2.17	13.7	1.97
	60 min		1.074	1,462	58	5.86	1.21	25.0	4.72

L-2071 cells previously growing on NCTC-135 medium with no added serum were incubated with doubly labeled HDL. At timed intervals, cells were harvested and the presence of [^{3}H]cholesterol and [^{125}I]apoprotein in the lipid extract were assessed by dual isotope liquid scintillation counting. The cell residue was analyzed for protein content and the results were listed as radioactive uptake per mg cell protein (cpm/mg). The ratio ^{3}H/^{125}I found in the cells after harvesting is normalized by dividing each ratio by the ^{3}H/^{125}I ratio of the original medium.

labeled lipoproteins are used as indicated by the results given in Table 9 and Fig. 1. An initial binding of intact ^{125}I-labeled lipoproteins to the cell membrane was indicated by the fact that the [^{3}H]cholesterol/^{125}I-labeled apoprotein ratio extrapolates to 1:1

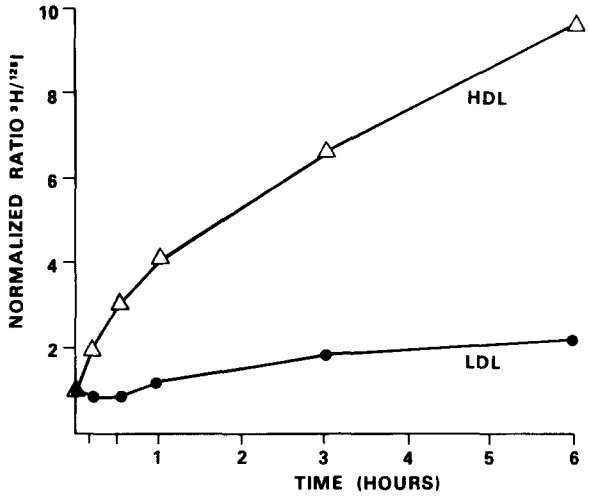

Fig. 1. [^{3}H]Cholesterol and ^{125}I-labeled apoprotein uptake by L-2071 fibroblasts from doubly labeled human lipoproteins. L-2071 cells grown on NCTC-135 with no serum were presented with various amounts of doubly labeled lipoprotein as described in the legend to Table 9. Dual isotope counting of ^{3}H and ^{125}I found in the washed, harvested cells was used to determine the relative amounts of cholesterol (^{3}H) and lipoprotein (^{125}I) incorporated into the cells. Note the immediate preferential uptake of cholesterol by cells from HDL as compared to the delayed uptake of cholesterol from the LDL-related lipoproteins. The absolute amounts of cholesterol and protein taken up after various time periods are given in Table 9.

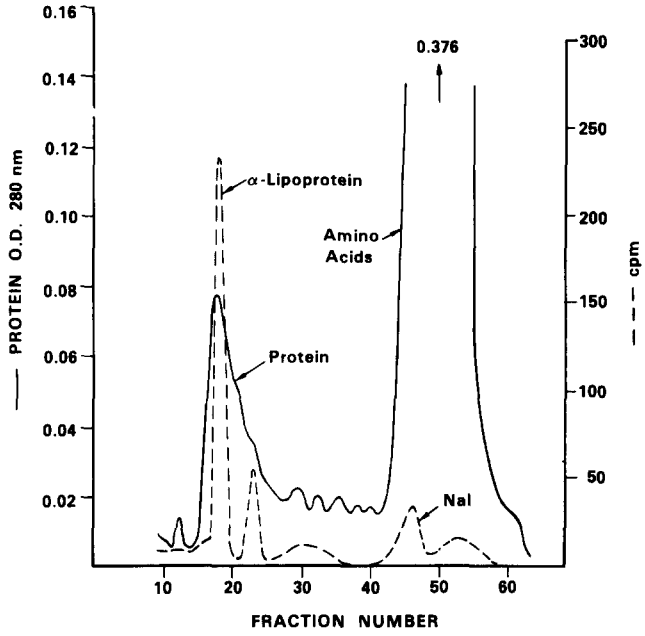

Fig. 2. Sephadex G-200 fractionation of spent medium from incubation of L-2071 cells in NCTC-135 supplemented with doubly labeled HDL (^{125}I-labeled apoprotein and [^{3}H]cholesterol). L-2071 fibroblasts were incubated with doubly labeled HDL (167 μg) in 20 ml of incubation medium for 24 hr, at which time 40% of the labeled cholesterol but only 2.7% of the labeled apoprotein moiety had been taken up by the cells. The pooled spent media from triplicate cultures were concentrated in a Diaflo apparatus using a PM-30 filter to about 20 ml, loaded on a Sephadex G-200 column (100 × 5 cm), and eluted with 0.005 M phosphate–0.4 M NaCl buffer at pH 7.0. Ten-ml fractions were collected and the protein was read at 280 nm. Most of the ^{125}I was eluted in the α-lipoprotein region, showing that the removal of cholesterol from the lipoprotein and transfer into the cell releases essentially unchanged apoprotein into the medium.

at zero time for both HDL and LDL. However, within 10 min, cholesterol is already preferentially removed from HDL (compared to apoprotein) in a ratio of 2:1 and this ratio progressively increased to 10:1 at 6 hr of incubation. Conversely, the $^{3}H/^{125}I$ ratios for the LDL uptake were quantitatively different from those for HDL. The $^{3}H/^{125}I$ uptake ratio for the LDL remained at approximately 1:1 for the first 30 min to 1 hr and had only increased to 2:1 at 6 hr of incubation. The transfer of cholesterol from LDL to cells was also a slower process than from HDL to cells. The varying uptake rates of cholesterol may be related to the varying degrees of uptake of the apolipoproteins. Analysis by Sephadex column chromatography showed that the HDL apoprotein remaining in the medium had the same characteristics as that added initially; therefore, loss of cholesterol was not accompanied by significant hydrolysis or breakdown of the apoprotein moiety. These results show that when radioactive cholesterol is transferred or exchanged between HDL and a cell, it occurs without internalization and degradation of the apoprotein moiety. This is in contrast to the transfer of cholesterol from LDL to the cell which, as shown by Brown and Goldstein (2–4), involves the internalization and degradation of the intact LDL molecule. The data in Fig. 1 do not imply the *net* transfer of cholesterol from HDL to the cell. In fact it has been shown that HDL-catalyzed efflux from the cell is about 3 times larger than influx under incubation conditions similar to those used in these experiments.

Studies of LDL binding and HDL binding to skin fibroblasts by Miller, Weinstein, and Steinberg (25) and Koschinsky, Carew, and Steinberg (26) also demonstrated the fundamental differences between the binding of these two lipoproteins. These differences include the observations that: *1*) the rates of internalization and degradation of ^{125}I-labeled HDL are very low relative to those of ^{125}I-labeled LDL; *2*) HDL does not share the ability of LDL to suppress cholesterol synthesis in peripheral cells; *3*) HDL binding is not affected by the presence of cholesterol and 7-ketocholesterol; and *4*) HDL uptake can be adequately accounted for by bulk endocytosis and absorptive endocytosis occurring randomly wherever HDL is found, while uptake of LDL is much too large to be accounted for in this way and, as shown by Brown and co-workers (2–4), involves a different mechanism.

The results of the present studies show that both lipoproteins were bound at specific, saturable sites and an excess of one lipoprotein did not displace the other. They also indicate that HDL and LDL are taken up at separate binding sites by fundamentally different mechanisms. LDL uptake involves lysomal processes, is inherently self-destructive, and is linked to regulation of cholesterol biosynthesis. In contrast, HDL binding does not involve the internalization of apoprotein and the chief function of its interaction with cells as revealed in previous studies (11) is probably the removal of cholesterol from cells by interactions which are basically similar to those described here.

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