Receptors for homologous plasma lipoproteins on a rat hepatoma cell line

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Abstract Hepatocytes express on their surfaces more than one class of receptors capable of mediating the internalization of lipoproteins. However, relatively little is known about the binding characteristics of hepatic receptors for various lipoproteins, about the regulation of the receptors, and about the consequences for intracellular lipid metabolism of uptake of lipoproteins via different classes of receptors. The aim of the present studies was to characterize the binding and degradation of various lipoproteins and their mutual competition for cellular processing. Since these kinds of studies may be more easily carried out in continuous established hepatoma cell lines than in nondividing primary hepatocyte cultures, we examined the lipoprotein receptor functions of a well differentiated rat hepatoma (H-35). Cells were grown to confluence in Eagle's minimal essential medium in 15% newborn calf serum. Medium then was changed to 15% lipoprotein-deficient serum for 44 hr before experiments. External binding of ^{125}I-labeled rat plasma and intestinal lymph lipoproteins was assessed at 4°C. Cellular uptake and degradation were assessed at 37°C. Lipoproteins were isolated by fixed angle or zonal ultracentrifugation or by heparin affinity column chromatography and characterized as to their lipid and apoprotein compositions. Labeled low density (LDL), high density (HDL_2), non-apoE-HDL, very low density lipoproteins (VLDL), and chylomicron remnants (CM-R) each manifested specific and saturable binding and degradation by the hepatoma cells. Competition experiments indicated that separate receptors were present for LDL, HDL_2, and CM-R. Most of HDL_2 appeared to be bound to the non-apoE-HDL receptor. Scatchard analyses provided different binding constants for the LDL and HDL receptors. EDTA (5 mM) and suramin (0.1 mM) inhibited binding at the LDL receptor, but not at the HDL receptor. At similar concentrations, suramin but not EDTA inhibited binding of CM-R. In contrast to the LDL receptor of human cells, where apoE is bound more avidly than apoB, LDL and apoE-containing lipoproteins appeared to be bound with equal avidity by the hepatoma LDL receptor, suggesting that this LDL receptor may differ from the human LDL receptor; alternatively, rat apoE may differ from its human counterpart either with respect to structure or with respect to how its interaction with cells is modulated by other lipoprotein components.—**Tamai., T., W. Patsch, D. Lock, and G. Schonfeld.** Receptors for homologous plasma lipoproteins on a rat hepatoma cell line. *J. Lipid Res.* 1983. **24:** 1568–1577.

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The liver is not only a major source of the lipoproteins circulating in the plasma, but it also participates in the removal of lipoproteins from plasma (1–13). High affinity uptake mechanisms of chylomicron remnants by the liver have been described by several investigators (14–16). In addition, livers of humans, dogs, rats, and pigs also can express surface receptors for LDL, especially under metabolic conditions when there is increased need for cholesterol by the liver (17–23). Both types of hepatic lipoprotein receptors have been detected in intact animals, perfused livers, liver membranes, and primary hepatocyte cultures whether studied as cell suspensions or as plated cells (18–23). A less specific lipoprotein receptor also has been described on porcine hepatocytes (24). Other cell types that express more than one lipoprotein receptor are macrophages and adrenal cells (25–27). Whether the various receptors mediate similar or different processes of the hepatocyte's intracellular metabolism of cholesterol is not known. Nor is it known how different lipoproteins may affect lipoprotein production. One of our long-term aims is to obtain such information in hepatocytes. However, primary hepatocyte preparations actively secrete lipoproteins into the culture medium at 37°C (11, 28). This makes interpretation of studies of lipoprotein uptake difficult because of dilution of tracer material by endogenous sources. To overcome these difficulties we have adopted a rat hepatoma cell line (H-35) which, although it retains several differentiated functions of hepatocytes (29–33), secretes greatly reduced amounts of lipids and apoproteins into the culture medium. We report here on the expression of three surface receptors on these cells that are capable of mediating the binding, internalization, and subsequent degradation of different classes of homologous lipoproteins.

Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein; Cm-R, chylomicron remnants; TMU, tetramethylurea; DME, Dulbecco's modified Eagle's medium; NBCS, newborn calf serum; LPDS, lipoprotein-deficient serum; MEM, minimal essential medium; apoA-I, apoprotein A-I; apoB, apoprotein B; apoC-III, apoprotein C; apoE, apoprotein E; $apoB_L$, large apoprotein B; $apoB_S$, small apoprotein B.

METHODS

Blood was obtained from the inferior venae cavae of female Sprague-Dawley rats weighing 250–400 g and collected in tubes containing 1 mg EDTA/ml of blood. Plasma was separated immediately by low speed centrifugation at 4°C and lipoprotein separation was started within 12 hr. VLDL was isolated by ultracentrifugation in a 60 Ti rotor (Beckman Instruments, Palo Alto, CA) at d 1.006 g/ml, 40,000 rpm, 16 hr, and 15°C. For preparation of LDL and HDL_1, the d 1.006 g/ml bottom fraction or whole plasma was adjusted to a nonprotein solvent density of 1.07 g/ml by addition of solid KBr and ultracentrifuged in a 60 Ti rotor at 40,000 rpm for 20 hr at 15°C. The top fraction was then removed and subjected to zonal ultracentrifugation in a 14 Ti rotor using a linear NaBr density gradient spanning the densities 1.00 to 1.30 g/ml. Running conditions were 140 min, 42,000 rpm, and 15°C (34). Two lipoprotein populations with peak elution volumes of 220 and 320 ml were obtained (**Fig. 1**). The faster floating population had zonal flotation properties similar to that of human LDL and was therefore designated LDL. The second slower floating population was designated HDL_1. The remaining HDL was isolated from the d 1.07 g/ml infranatant, which was adjusted to d 1.21 g/ml by solid KBr. Ultracentrifugation was for 24 hr at 40,000 rpm at 4°C in a 60 Ti rotor. Both VLDL and HDL were washed once by ultracentrifugation at d 1.006 and 1.21 g/ml, respectively. HDL as isolated by this conventional procedure produced one major lipoprotein population upon zonal ultracentrifugation with flotation properties similar to human HDL_2 (35). Therefore it was designated as HDL_2. ApoE-containing HDL was separated from non-apoE-HDL by passing HDL_2 obtained as above over a heparin-Sepharose CL-4B affinity column (36). Fourteen percent of recovered protein was retained on the column (apoE-HDL); >95% of the loaded protein was recovered. Chylomicron remnants (CM-R) were prepared as follows. Rats were given 1.5 ml of corn oil via gastric tube. One to 2 hr later, under ether anesthesia, cisternae chyli were cannulated and lipemic lymph was collected for ~24 hr in tubes containing 0.5 ml of 0.1 M EDTA, 10 μl of 1 mg/ml chloramphenicol, 20 μl of 2 mg/ml gentamycin, 10 μl of 80 mg/ml NaN_3, and 10 μl of 5 mg/ml phenylmethyl sulfonyl fluoride (37) under sterile conditions. Chylomicrons were floated in a SW27 rotor (3×10^6 g-min, 10°C), recovered by slicing the tubes, and injected into functionally hepatectomized rats that had been fasted for 24 hr prior to surgery. After ~60 minutes the recipient rats were exsanguinated via their inferior venae cavae and EDTA plasmas were prepared, brought to a calculated background density of 1.019 g/ml with solid KBr, and ultracentrifuged in a 50.3 rotor (2×10^8 g-min, 10°C). Chylomicron remnants were harvested by tube slicing. Chemical compositions of lipoproteins were determined by measuring protein (38), triglycerides, and free and esterified cholesterol (Enzymatic Kits, Boehringer Mannheim, Indianapolis, IN), and phospholipids (39). Proportions of various apoproteins of lipoproteins were

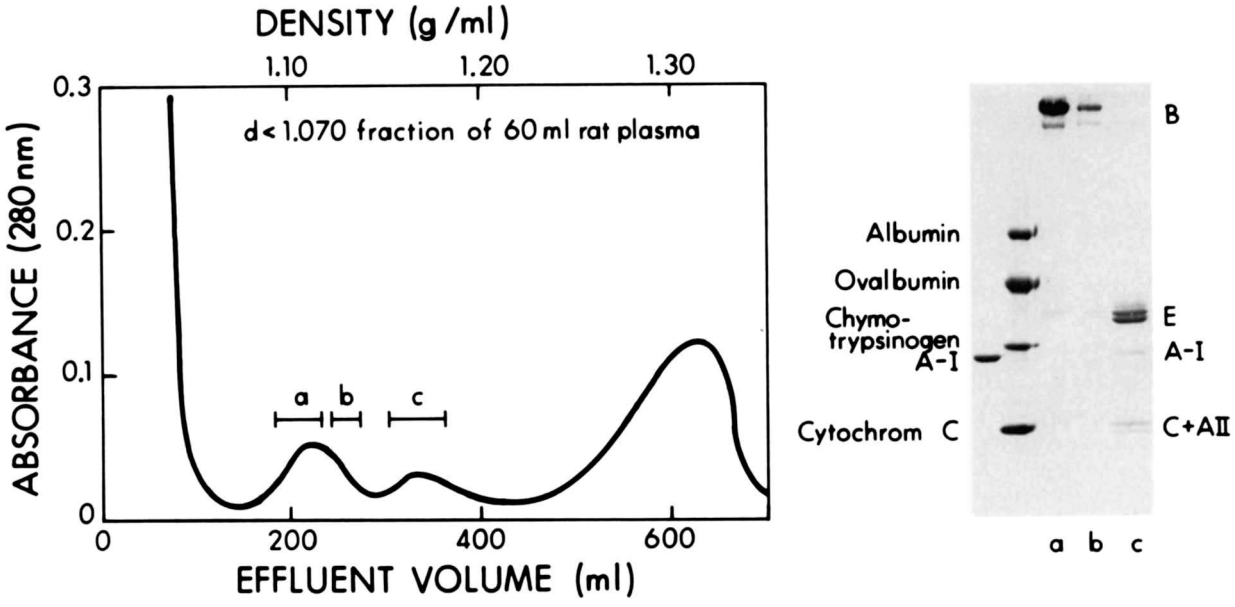

Fig. 1. Isolation of LDL (a, b) and HDL_1 (c) subfractions by rate zonal ultracentrifugation from the d < 1.070 plasma fractions which were obtained from 60 ml of rat plasma. Apoproteins of respective lipoprotein fractions separated by 0.1% SDS-PAGE electrophoresis are shown on the right side of the figure. Lanes from left to right represent isolated rat apoprotein A-I, the indicated molecular weight standards, and samples a, b, and c, respectively. Apoproteins B, E, A-I, C, and A-II are identified.

assessed by electrophoresis in 3.0% or 10% single concentration polyacrylamide gels, or 3–20% linear gradient gels (40). Coomassie blue-stained gels were scanned and areas of dye uptake were integrated as described (41). Protein insoluble in 4.2 M tetramethylurea (TMU) was used as a measure of apoprotein B content (42).

Lipoproteins were radiolabeled with iodine monochloride (43). Specific activities were between 150 and 300 cpm/ng of protein. Lipid extractable (44) radioactivities averaged 20, 13, 8, 4, and 4% of total radioactivity in CM-R, VLDL, LDL, non-apoE-HDL, and HDL_2, respectively. Lipoproteins were used within 3 weeks of isolation and were stored in antibiotics and protease inhibitors (45). Lipoproteins used in cell interaction experiments were dialyzed against 0.154 M NaCl containing 1 mM EDTA, pH 7.4, before use on cells. The amount of EDTA added to incubation media by these lipoprotein solutions made media <0.14 mM in EDTA. $[Ca^{2+}]$ of media was 1.8 mM.

H-35 rat hepatoma cells (29–33) were maintained in Dulbecco's modified Eagle's medium (DME, Gibco, Grand Island, NY) supplemented with 15% newborn calf serum (NBCS), 0.62 μg/ml amphotericin B, penicillin (100 U/ml), and streptomycin (100 mg/l). Stock cultures were passaged weekly by seeding 2×10^6 cells per 100-mm dish in 8 ml of media. Confluent monolayers were dislodged by the addition of Ca^{2+}–Mg^{2+}-free saline containing 0.05% trypsin in EDTA. Binding experiments were done between the 3rd and 10th passage after thawing of cells. For binding experiments $4–5 \times 10^5$ cells were seeded on 35-mm dishes on day 1. NBCS was replaced by 15% lipoprotein deficient serum (LPDS) on day 3, and on day 5 of culture, cells were incubated with various concentrations of iodinated lipoproteins in minimal essential medium (MEM) containing 25 mM Hepes and 7% LPDS, for 4 hr at 4°C. At the end of incubation, dishes were washed four times with ice-cold Tris-HCl (0.1 M, pH 7.4) containing 0.2% albumin followed by two washes in the absence of albumin. Cells then were dissolved in 1 N NaOH overnight. Aliquots were assayed for radioactivity and cellular protein. Nonspecific binding was determined by addition of a 20-fold excess of cold lipoprotein along with the tracer. Binding constants were determined by graphic analysis of Scatchard plots of data obtained in competitive displacement assays (46).

Studies were also done at 37°C to determine uptake and degradation of various lipoprotein classes (47). Incubations were carried out with increasing doses of radiolabeled lipoproteins in MEM containing 7% LPDS for 4 hr. At the end of incubation, media were removed for determination of trichloroacetic acid-soluble material, which was used as the measure of intracellular lipoprotein-protein degradation. Cells then were quickly cooled to 4°C, washed, and dissolved in 1 N NaOH, to determine ^{125}I-labeled lipoprotein associated with cells.

Cellular DNA content was determined by the method of Kapuscinski and Skoczylas (48). Accumulation of apoproteins E, C-III, and B in media was measured by published radioimmunoassay procedures (11, 49, 50).

RESULTS

The lipoprotein-cell interaction studies to be described were performed when cells had reached confluence and been maintained in 15% LPDS for 44 hr, in order to minimize any effects of variations of cell density and cholesterol availability on binding characteristics.

To determine whether the hepatoma cells secreted any lipoproteins, apoproteins were measured in the media of confluent cells after 48 hr of incubation as indices of lipoprotein secretion. Media contained 240 ng/ml of apoE, 36 ng/ml of $apoC\text{-}III_3$, and 47 ng/ml of apoB. These values represent ~1/15 of the amount of apoproteins accumulating in primary rat hepatocyte cultures incubated under similar conditions (11, 28).

The chemical compositions of representative lipoprotein isolates used in the cell interaction studies (**Table 1**) are fairly typical of compositions reported previously (12, 36, 51–56). Apoprotein contents of lipoprotein fractions (**Table 2** and **Table 3**) also resemble previously reported results (41, 53, 55, 56). The differences in the distributions of $apoB_L$ and $apoB_S$ in various lipoproteins suggest that these lipoprotein fractions may contain differing proportions of hepatic and intestinal particles. Rat apoE migrates as more than one band in SDS polyacrylamide gels. The molecular basis for this is not clear (41). Also it

TABLE 1. Chemical composition of lipoproteins used in hepatoma binding studies

Lipoprotein Fractions	Lipoprotein Components				
	Protein	PL	FC	CE	TG
	% of mass				
CM	1.9	11.6	1.0	0.2	85.3
CM-R	7.1	18.8	0.1	2.2	71.8
VLDL	8.6	14.0	3.8	3.2	70.2
LDL	25.3	26.0	16.8	25.8	6.2
HDL_1	31.1	25.2	8.3	18.5	17.0
HDL_2	39.1	28.2	4.7	23.9	4.0

Chylomicrons (CM) were isolated from intestinal lymph; CM remnants (CM-R) were produced in functionally hepatectomized rats; VLDL was isolated from rat plasma at d 1.006 g/ml; plasma LDL is made up of fractions a and b of zonal runs (Fig. 1); plasma HDL_1 is fraction c (Fig. 1); plasma HDL_2 was isolated at d 1.070–1.210 g/ml. HDL_2 floats at the same rate as does human HDL_2 in the zonal ultracentrifuge (41). Results are representative of three or four isolates of each lipoprotein fraction.

TABLE 2. Apoprotein compositions of rat lipoproteins

Lipoprotein Fractions	Apoprotein Components				
	B[a]	E	A-I	C + A-II	A-IV
	% stained area				
CM	24	10	23	39	4
CM-R	8	64	2	20	6
VLDL	45	39	n.d.	16	n.d.
LDL	>95	<3	n.d.	n.d.	n.d.
HDL_1	9	75	14	2	n.d.
HDL_2	n.d.	17	61	11	11
ApoE-HDL	n.d.	87	6	7	n.d.
Non-apoE-HDL	n.d.	n.d.	71	21	8

[a] ApoB result is % of lipoprotein mass as tetramethylurea-insoluble protein; proportions of apo'sE, A-I, C + A-II, and A-IV were determined by scanning of stained SDS-polyacrylamide gels (e.g., Fig. 1). For abbreviations, see Table 1. n.d., none detected.

should be pointed out that the HDL_1 in this study differs from the fraction designated as HDL_1 in an earlier study (41) with respect to flotation properties and chemical and apoprotein composition. The previously described HDL_1 was found only after feeding a high carbohydrate diet to female rats, whereas the HDL_1 in this study was isolated under different conditions from rats fed Purina Lab Chow. Furthermore, HDL_1 and apoE-HDL that were isolated under different conditions were not identical in composition (Table 2).

In binding experiments performed at 4°C, saturable high affinity binding sites were detectable for ^{125}I-labeled LDL, HDL_2, non-apoE-HDL, VLDL, and CM-R (**Fig. 2** and **Table 4**). The binding of ^{125}I-non-apoE-HDL performed at a later date was even higher than that of ^{125}I-HDL_2. Specific saturable cellular uptake (binding plus internalization) and degradation also were detected for ^{125}I-LDL, ^{125}I-HDL_2, and ^{125}I-VLDL when incubations were performed at 37°C (**Fig. 3**) suggesting that binding led to intracellular processing of the lipoproteins.

To assess whether binding was occurring at more than one receptor site, competitive displacement assays were performed at 4°C (**Fig. 4, Table 5** and **Table 6**). VLDL, LDL, and HDL_1 were able to compete with ^{125}I-LDL for binding to cells. HDL_2 was much less active. When results

TABLE 3. Apoprotein B subspecies in rat lipoproteins

Lipoprotein Fractions	ApoB Components	
	B_L	B_S
	% area	
CM	<2	>98
CM-R	10	90
VLDL	72	28
LDL	93	7
HDL_1	15	85

Results are dye uptake areas determined on 3.0% or 3.0–20% gradient SDS-polyacrylamide gels. B_L, large apoB, B-100 or P-I; B_S, small apoB, B-48 or P-III (56).

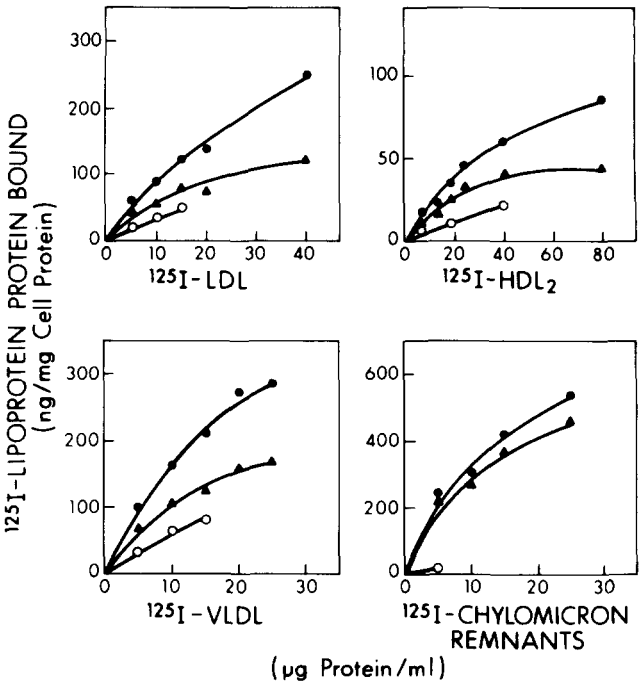

Fig. 2. Saturation curves for binding at 4°C of rat ^{125}I-LDL, ^{125}I-HDL_2, ^{125}I-VLDL, and ^{125}I-chylomicron remnants. Cells were grown in 15% LPDS for 44 hr. The indicated concentrations of rat ^{125}I-lipoproteins were added for 4 hr at 4°C in the absence (●) or presence (○) of 20-fold concentrations of the respective unlabeled rat lipoproteins. Specific binding (▲) was calculated from the differences between total (●) and nonspecific binding (○). Since nonspecific binding was not evaluated at the higher doses of ^{125}I-labeled lipoproteins (due to difficulty in acquiring the large amounts of lipoproteins needed), the specific binding curves at the higher doses represent extrapolations. Results are representative of two or three individual experiments carried out in duplicate or triplicate dishes 3–6 months apart. Means of all dishes had coefficients of variation of 6–16%.

TABLE 4. Effect of competitors on binding of ^{125}I-non-apoE-HDL

Concentration of ^{125}I-non-apoE-HDL in Culture Medium	^{125}I-non-apoE-HDL Bound		
	Total	Nonspecific	Specific
μg/ml	*ng/mg cell protein*		
5	70 ± 3	33 ± 1	37
10	116 ± 2		70
30	228 ± 2	135 ± 10	93
50	297 ± 27		88

Cells were grown in 15% LPDS for 44 hr. The indicated concentrations of rat ^{125}I-non-apoE-HDL were added to 4 hr at 4°C in the absence or presence of 20-fold concentrations of the unlabeled non-apoE-HDL. Specific binding was calculated from the differences between total and nonspecific binding; linear extrapolations were used to obtain some nonspecific binding values and then these were used to calculate specific values. This experiment was carried out in triplicate dishes, results are means ± 1 SD. Similar experiments were carried out on two other occasions, with compatible results (means of all these experiments had coefficients of variation between 5.6 and 16.8%).

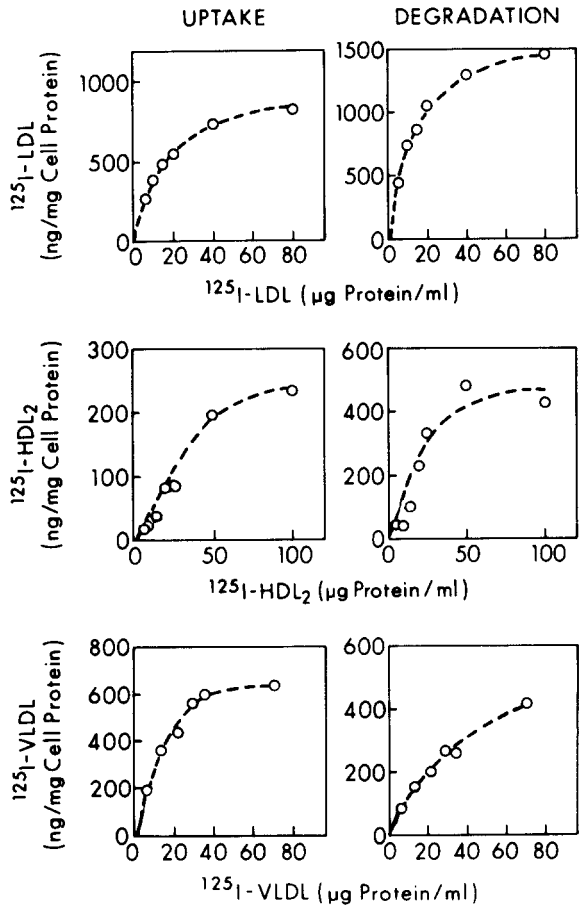

Fig. 3. Saturation curves for uptake (surface binding plus internalization) and degradation of rat ^{125}I-LDL, ^{125}I-HDL$_2$, and ^{125}I-VLDL by hepatoma cells; incubation was at 37°C for 4 hr. Cells were grown in 15% LPDS (○ – – – ○) for 44 hr. Results are means of triplicate dishes per point. Specific uptake and degradation have been calculated as the difference between total uptake and degradation and uptake and degradation in the presence of 20-fold excess concentrations of the respective unlabeled rat lipoproteins. Nonspecific uptake and degradation represented <20% of total uptake and degradation. Coefficients of variation of totals averaged 12%.

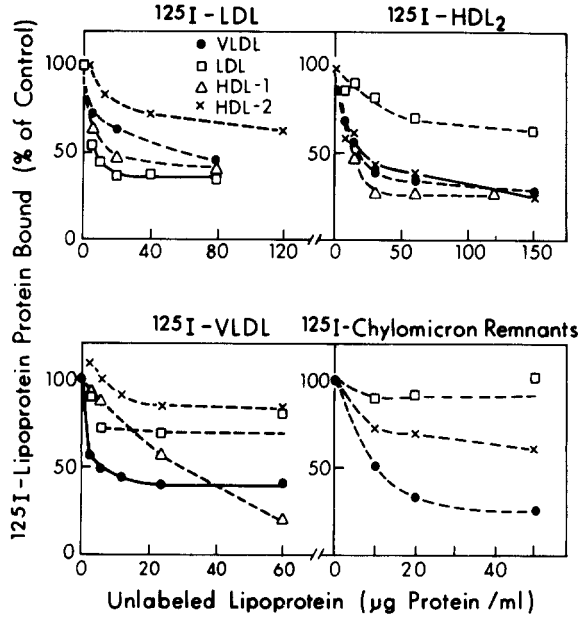

Fig. 4. Competition of various rat lipoproteins against each other for binding to H-35 hepatoma cells grown in LPDS. Displacement curves for ^{125}I-LDL, ^{125}I-HDL$_2$, ^{125}I-VLDL, and ^{125}I-chylomicron remnants (CM-R) by rat unlabeled VLDL (●), rat LDL (○), rat HDL$_1$ (△), and rat HDL$_2$ (×) are shown. Dishes contained constant amounts of ^{125}I-LDL (3 μg/ml), ^{125}I-HDL$_2$ (15 μg/ml), ^{125}I-VLDL (15 μg/ml), ^{125}I-CM-R (8 μg/ml), and various amounts of unlabeled lipoprotein. One hundred percent binding for ^{125}I-LDL, ^{125}I-HDL$_2$, ^{125}I-VLDL, and ^{125}I-Cm-R were 51 ± 4, 28 ± 7, 183 ± 11, 246 ± 3, respectively, in ng lipoprotein-protein/mg cell protein (mean ± SD).

were expressed based either on the mass of apoE in the lipoproteins or based on moles of apoE per particle, the VLDL and HDL$_1$ competition curves became identical with each other and with the LDL displacement curve, i.e., the apoE of VLDL and HDL$_1$ was equally, but not more, effective on a mass basis than was LDL-apoB in competing with ^{125}I-LDL for binding. The curve produced by HDL$_2$ remained to the right and above the LDL curve.

Binding of ^{125}I-HDL$_2$ was equally well inhibited by HDL$_2$, HDL$_1$, and VLDL, but much less so by LDL (Fig. 4, Table 6). Thus, LDL and HDL$_2$ probably interacted with different cellular receptors. To assess whether the abilities of HDL$_1$ and VLDL to compete with ^{125}I-HDL$_2$ were due to the sharing of apoE by all of these lipoproteins, ^{125}I-non-apoE-HDL (10 μg/ml) was incubated at 4°C in the absence and in the presence of the following

TABLE 5. Binding of ^{125}I-non-apoE-HDL in the presence of various competitor rat lipoproteins

Unlabeled Competitor Lipoproteins	Concentration of Unlabeled Competitor Lipoprotein (μg/ml)					
	0	10	20	40	80	200
Non-apoE-HDL	100	82 ± 6	68 ± 4	55 ± 3	37 ± 1	36 ± 6
LDL	100	112 ± 7	133 ± 13	88 ± 2	86 ± 9	
HDL$_2$	100	90 ± 4	77 ± 10	63 ± 5	52 ± 4	
ApoE-HDL	100	88 ± 8	88 ± 8	73 ± 5	114 ± 5	

Cells were grown in 15% LPDS for 44 hr. Ten μg/ml of rat ^{125}I-non-apoE-HDL was incubated for 4 hr at 4°C with the indicated amounts of various unlabeled lipoproteins. Results are percentage of control (100% = 104 ng of ^{125}I-non-apoE-HDL bound/mg cell protein) (mean ± 1 SD, n = 3).

TABLE 6. Summary of results of competition assays

Competing Lipoproteins	Labeled Lipoproteins				
	LDL	HDL_2	Non-apoE-HDL	CM-R	VLDL
LDL	4	150	1* (23%)	2* (0%)	8
HDL_1	10	10	9	NT	15
HDL_2	120	12	35	70	3* (29%)
Non-apoE-HDL	NT	NT	20	NT	NT
ApoE-HDL	NT	NT	1* (21%)	NT	NT
VLDL	33	13	10	9	4

Results are taken from Fig. 4 and other experiments. Experimental dishes contained constant amounts of ^{125}I-LDL (3 μg/ml), ^{125}I-HDL_2 (15 μg/ml), ^{125}I-non-apoE-HDL (10 μg/ml), ^{125}I-CM-R (8 μg/ml), or ^{125}I-VLDL (15 μg/ml), and the indicated amounts of unlabeled lipoproteins. Vertical columns can be compared with each other since they represent single or identical experiments. Numbers represent doses (in μg/ml) of competitor lipoproteins required to obtain 50% of maximum inhibition of binding of labeled lipoproteins produced by the underlined competitor. For example, in the case of competition assay of ^{125}I-VLDL (fifth column), the 4 in the sixth row (VLDL) represents the dose of unlabeled VLDL which produced 50% of the maximum inhibition of ^{125}I-VLDL. The 8 and 15 in the first (LDL) and second (HDL_1) rows represent the doses of respective unlabeled lipoproteins which produced 50% of the maximum inhibition of ^{125}I-VLDL by unlabeled VLDL. The 29% of (3*) in the third row (HDL_2) represents 29% of the maximum inhibition of ^{125}I-VLDL produced by the maximum dose of unlabeled HDL_2 concentration used (60 μg/ml).

1*, Maximum unlabeled lipoprotein 80 μg/ml; 2*, maximum unlabeled lipoprotein 50 μg/ml; NT, not tested.

competing unlabeled lipoproteins: LDL, non-apoE-HDL, apoE-HDL, HDL_2 and HDL_1, and VLDL (10 to 80 μg/ml for each competitor). LDL and apoE-HDL competed poorly (Tables 5 and 6) while non-apoE-HDL and HDL_2 competed about equally well with ^{125}I-non-apoE-HDL. The equal competitiveness of non-apoE-HDL and HDL_2 suggest that HDL_2 was capable of being bound to the same receptor as non-apoE-HDL. Indeed this is not surprising, since ~86% of HDL_2 protein was not bound to the heparin colums and HDL_2 contained only 17% apoE. HDL_1 and VLDL also competed. The competitiveness of HDL_1 and the lack of competitiveness of apoE-HDL may be due to differences in the structures or in the preparation of those fractions. Others have noted that ultracentrifugation and column chromatography have differing effects on the cellular reactivity of lipoproteins (57). The competitiveness of VLDL vs. ^{125}I-non-apoE-HDL is difficult to explain in terms of apoprotein contents since these two lipoproteins share only the smaller apoproteins C and/or A-II, which are thought not to be involved in lipoprotein binding to cells; however it is worth noting that VLDL seemed to compete vs. all labeled lipoproteins. In any case, it appears that a non-apoE-HDL receptor is present on this hepatoma line.

Interaction of ^{125}I-labeled CM-R with cells was inhibited by CM-R (Fig. 6), and VLDL; HDL_2 inhibited partially and LDL minimally (Fig. 4, and Table 6). This suggests that CM-R may be bound to a receptor that is distinct from both the LDL and HDL_2 receptors.

Binding of ^{125}I-VLDL was inhibited by VLDL, HDL_1, and LDL (Fig. 4). Also, VLDL inhibited the binding of ^{125}I-CM-R, ^{125}I-LDL, and ^{125}I-HDL_2. Thus, VLDL may be bound to the HDL_2, LDL, and the CM-R receptor. The ability of HDL_1 to compete vs. several labeled lipoproteins suggests that it too was bound to more than one receptor.

Binding constants, determined in competitive displacement assays, are shown in **Fig. 5** and **Table 7.** Two binding sites for VLDL was resolved by graphic analysis of Scatchard plots (58), but only one binding site for HDL_2 and another one for LDL were apparent. The receptor for LDL exhibited higher affinity and lower capacity than the receptor for HDL_2. VLDL was bound to one receptor the binding constants of which resembled that of LDL and the other that of HDL_2.

Lipoprotein binding of hepatoma cells was selectively affected by the addition of EDTA (59) or suramin (60, 61) to culture media during the 4°C binding experiments (**Fig. 6**). Specific binding of ^{125}I-LDL was completely abolished at EDTA concentration of 20 mM, and greatly reduced at suramin concentration of 0.1 mM. ^{125}I-labeled CM-R binding was much less sensitive to inhibition by EDTA but it was sensitive to suramin. Cooper et al. (62) also reported that CM-R binding to rat liver membranes was inhibited only moderately by 10 mM EDTA. HDL_2 and non-apoE-HDL were much less affected by either agent. These results support the distinctiveness of binding of LDL, CM-R, and HDL_2 to cellular binding sites.

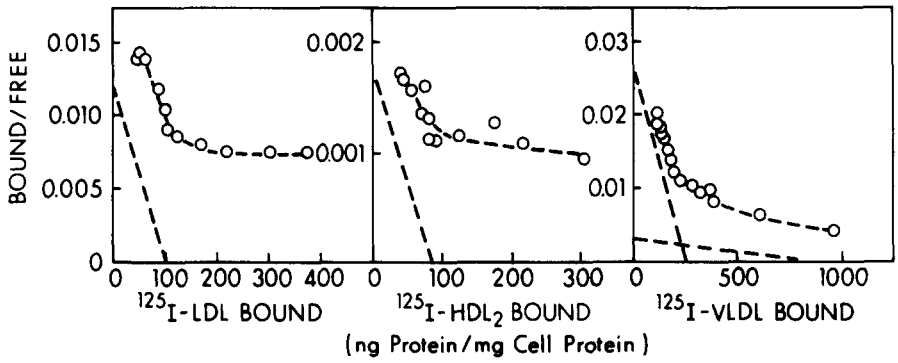

Fig. 5. Scatchard plots of binding of ^{125}I-LDL, ^{125}I-HDL$_2$, and ^{125}I-VLDL. Cells were grown in 15% LPDS (○ – – – ○) for 44 hr. Constant amounts of ^{125}I-LDL (7 μg of LDL protein/ml), ^{125}I-HDL$_2$ (20 μg/ml), or ^{125}I-VLDL (5 μg/ml) were added along with increasing concentrations of their respective unlabeled homologues. Doses for LDL ranged from 0 to 150 μg/ml, for HDL$_2$, from 0 to 600, and for VLDL, from 0 to 300. Incubations were at 4°C for 4 hr. Heavy curved lines show Scatchard plots of total binding. Specific binding was obtained by subtraction of labeled lipoproteins bound in the presence of maximum doses on unlabeled lipoproteins, according to Chamness and McGuire (58). Specific binding data were used to construct the lighter straight lines which yield the Kd's and binding capacities presented in Table 6.

DISCUSSION

Rat H-35 hepatoma cells appear to possess on their surfaces sites for specific binding of homologous lipoproteins. One set of receptors binds LDL with relatively high affinity; binding at this site is inhibited in the presence of <5 mM EDTA and <0.1 mM suramin in the medium. Another set of receptors binds HDL$_2$ at lower affinity. Binding at this second site is much less inhibited by suramin and EDTA. Non-apoE-HDL also may be bound at the HDL$_2$ site but LDL does not interact with this receptor. Chylomicron remnants seem to be bound at a receptor that may be distinct from both the LDL and HDL$_2$ receptors. HDL$_1$ and VLDL appear to be capable of interacting with each of these receptors.

Our experiments do not provide direct information on the apoprotein moieties of lipoproteins that are bound by the receptors, however it is likely that apoB$_L$, the major apoprotein of LDL, mediates the binding of LDL to the LDL bind site in hepatoma cells. The small amount of apoE in our LDL preparations (about 3% of total protein, likely representing a contamination with HDL$_1$) probably was of minor importance in LDL binding, because purified HDL$_1$, the protein of which consists of 75% apoE, was no more effective than LDL on a mass basis in competing with ^{125}I-LDL for receptor occupancy. If binding of LDL were due to contamination with HDL$_1$, purified HDL$_1$ should have been 30 times more effective than LDL (on a protein mass basis) in competing for the LDL binding site.

It should be noted that the apoE on VLDL and HDL$_1$ was no more effective than LDL in competing with ^{125}I-LDL. Innerarity, Pitas, and Mahley (63) have reported similar binding affinities for rat apoE-containing lipoproteins and rat LDL to rat fibroblasts. Since apoE-containing lipoproteins are usually more effective than LDL in binding to the apoB,E receptor of nontransformed human cells (64), these data suggest that the rat LDL receptor may differ from the human apoB,E receptor. Alternatively, rat apoE may differ from human apoE in its binding

TABLE 7. Binding constants for rat lipoproteins to rat hepatoma cells

Labeled Lipoprotein	K_d		Binding Capacity	
	μg/ml	*[M]*	*μg/mg cell*	*particles/cell*
^{125}I-LDL	8.24	2.73×10^{-9}	99	20,500
^{125}I-HDL$_2$	46.4	1.29×10^{-7}	84	89,500
^{125}I-VLDL	9.3	4.65×10^{-10}	247	21,500
	243.0	1.23×10^{-8}	818	71,300

K_d's and binding capacities were determined from the Scatchard plots of binding data as described in Methods and in Fig. 5. For the calculations, molecular weights of LDL, HDL$_2$, and VLDL were assumed to be 3×10^6, 0.36×10^6, and 20×10^6 daltons, respectively. Protein contents of LDL, HDL$_2$, and VLDL were 8.6, 24.1, and 39.1%, respectively (Table 1).

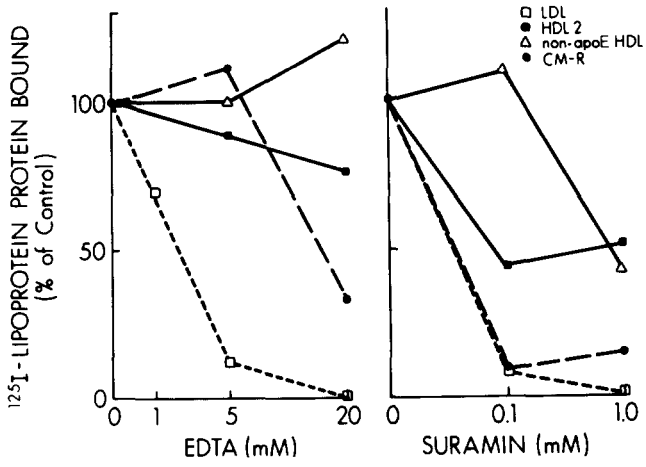

Fig. 6. The specific bindings of ^{125}I-LDL, ^{125}I-HDL$_2$, ^{125}I-non-apoE-HDL, ^{125}I-chylomicron remnants (Cm-R) to hepatoma cells were measured at 4°C as described in Methods, except that the incubation medium contained the indicated concentrations of EDTA or suramin and 7% lipoprotein deficient serum (LPDS). Dishes contained constant amounts of ^{125}I-LDL (Experiment I: 4 μg/ml, Experiment II: 15 μg/ml), ^{125}I-HDL$_2$ (15 μg/ml), ^{125}I-non-apoE-HDL (5 μg/ml), ^{125}I-CM-R (4.5 μg/ml). Nonspecific binding was assessed in the presence of 20-fold excess of the respective (LDL, HDL$_2$, non-apoE-HDL) nonradiolabeled lipoproteins; a 4-fold excess of nonradiolabeled CM-R was used. Each number represents the mean ± SD of triplicate determinations. Zero dose specific binding values were 73, 644, 71, 56, and 27 ng/mg cell protein for ^{125}I-LDL (Experiment I and Experiment II), ^{125}I-HDL$_2$, ^{125}I-non-apoE-HDL, and ^{125}I-CM-R, respectively.

characteristics, either because of some structural difference or because apoC-III or other lipoprotein components may modulate binding of apoE differently in rat and human lipoproteins (63). Obviously, more work is needed on this point.

HDL$_2$ (isolated between densities 1.07 and 1.21 g/ml) was less effective than HDL$_1$ in competing for ^{125}I-LDL binding, even when HDL$_2$ was corrected for its relatively small contents of apoE. This indicates that the mass of apoE in these lipoproteins is not the sole determinant of interaction with the LDL binding site on hepatoma cells. Rather it is the exposure of the appropriate apoE domains that may be important and this may be different on HDL$_1$ and HDL$_2$. An analogous situation exists for apoB where differences in the immunoreactivity of apoB occur within human VLDL subfractions and LDL (65). Whether differences in the exposure of any given apoprotein on lipoproteins are due to the presence of other "masking" apoproteins or lipids, or due to differences in the conformation of the given apoprotein on various lipoproteins, is not clear (66).

Binding of CM-R appears to be mediated by apoE, since CM-R, VLDL, and HDL$_2$ each inhibited ^{125}I-labeled CM-R binding and apoE is shared by these lipoproteins. Yet, the EDTA, suramin, and the competition experiments suggest that the majority of the HDL$_2$ particles and the Cm-R particles seem to be bound at different receptors.

Non-apoE-HDL is bound to a receptor that is clearly distinct from the LDL receptor. Since virtually no apoE is present in these particles, the cellular recognition must reside elsewhere, perhaps on apoA-I. A "lipoprotein receptor" with similar properties has been described on pig hepatic membranes (23). Both the pig hepatocyte and rat hepatoma "lipoprotein receptors" bind other lipoproteins as well. It is likely that HDL$_2$ may be bound in part to this receptor and in part to the chylomicron receptor. Clearly, additional studies are needed to define the apoprotein specificities of lipoprotein surface receptors, their structures, and regulation of expression and synthesis. Nevertheless, the data presented here suggest that the hepatoma cell line may be of aid in understanding receptor structure and physiology and the role of individual receptor pathways in the regulation of cellular, lipid, and lipoprotein metabolism.

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