Characterization of lipoprotein receptors on rat Fu5AH hepatoma cells

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Abstract The rat hepatoma cell line Fu5AH has the unusual property of accumulating massive amounts of cholesteryl ester upon incubation with hypercholesterolemic serum, and especially when incubated with beta-very low density lipoproteins (β-VLDL) from cholesterol-fed dogs. The present study was designed to identify and characterize the lipoprotein receptors that mediate the cholesteryl ester accumulation. The β-VLDL and cholesterol-induced apolipoprotein (apo) E-containing high density lipoproteins (apoE HDL_c) bound to Fu5AH cells with very high affinity ($K_d \simeq 10^{-10}$ M), whereas low density lipoproteins (LDL) bound with unusually low affinity ($K_d \simeq 10^{-8}$ M). Receptor binding activity of ^{125}I-labeled β-VLDL, ^{125}I-labeled apoE HDL_c, and ^{125}I-labeled LDL was abolished by incubation in the presence of an excess of unlabeled LDL or of a polyclonal antibody to the bovine adrenal apoB,E(LDL) receptor. The receptors were completely down-regulated by preincubating Fu5AH cells with β-VLDL, but much higher levels of β-VLDL were required than for down-regulation of fibroblast apoB,E-(LDL) receptors. Receptor binding was abolished by reductive methylation of the lysyl residues of the apolipoprotein of the β-VLDL and by an apoE monoclonal antibody (1D7) that blocks receptor binding. The Fu5AH receptor was further characterized by using the bovine adrenal apoB,E(LDL) receptor antibody. A single protein ($M_r \simeq 130{,}000$) was identified in Triton extracts of whole cells, and two proteins ($M_r \simeq 130{,}000$ and 115,000) were found in Fu5AH cell membranes disrupted by homogenization. The $M_r \simeq 115{,}000$ protein was released from the membranes and did not react with an antibody to the carboxyl-terminal (cytoplasmic) domain of the apoB,E(LDL) receptors. These studies indicate that Fu5AH cells express apoB,E(LDL) receptors that have unusually low affinity for apoB-containing lipoproteins, require large amounts of cholesterol to induce down-regulation, and are susceptible to specific proteolysis in cell homogenates. These apoB,E(LDL) receptors are responsible for the receptor-mediated uptake of β-VLDL and chylomicron remnants by Fu5AH cells.—**Friedman, G., M. E. Wernette-Hammond, D. Y. Hui, R. W. Mahley, and T. L. Innerarity.** Characterization of lipoprotein receptors on rat Fu5AH hepatoma cells. *J. Lipid Res.* 1987. **28:** 1482—1494.

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The rat hepatoma cell line Fu5AH is a fascinating cell line that has been used extensively by Rothblat and his associates to study cholesterol metabolism (1–8). Incubation of these cells with hypercholesterolemic rabbit or monkey serum resulted in the accumulation of cellular cholesteryl esters, whereas incubation with normal serum did not (1, 2). The cholesteryl ester accumulation in the Fu5AH cells was much greater than that observed with mouse L cells or with rabbit or monkey aortic smooth muscle cells (1, 3). Moreover, the cholesteryl esters accumulated in the cytoplasm as droplets, causing the cells to resemble foam cells (4). The lipoproteins floating at $d < 1.006$ g/ml, beta-very low density lipoproteins (β-VLDL), caused the greatest cellular accumulation (3).

The Fu5AH cells have also been used to study the mechanism of cholesterol efflux (5–8). When Rothblat, Bamberger, and Phillips (8) compared the half-time of cholesterol efflux from Fu5AH cells and from 12 other types of cells, including macrophages, fibroblasts, endothelial cells, and other liver cells, the Fu5AH cells exhibited the most rapid rate of cholesterol loss. Additional studies demonstrated that this accelerated cholesterol efflux was a property of the plasma membranes of the Fu5AH cells (8). Therefore, these cells possess an exaggerated ability both to take up hypercholesterolemic lipo-

Abbreviations: β-VLDL, beta-very low density lipoproteins; LDL, low density lipoproteins; apoE HDL_c, cholesterol-induced high density lipoprotein containing apolipoprotein E; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; MEM, minimal essential medium; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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proteins and to lose cholesterol stores in the presence of a suitable cholesterol acceptor in the medium.

Pattnaik and Zilversmit (9) demonstrated that chylomicron remnants were taken up and degraded by Fu5AH cells in culture. The properties of this cellular uptake suggested that the process was receptor-mediated. Further experimentation demonstrated that chylomicron remnants were catabolized by a receptor-mediated process, indicating that these cells possess receptors capable of interacting with chylomicron remnants (9).

The present study was designed to investigate the characteristics of the lipoprotein receptors on Fu5AH cells. It was postulated that Fu5AH cells may possess a distinct chylomicron remnant receptor because of their hepatic origin and because these cells bind and internalize chylomicron remnants (10, 11). Alternatively, it was thought that Fu5AH cells, which are similar to mouse macrophages in their ability to become foam cells, may possess an unusual apolipoprotein (apo) B,E(LDL) receptor, similar to that found on mouse peritoneal macrophages (12). The results of this study show that the only type of functional lipoprotein receptor on Fu5AH cells is an unusual apoB,E(LDL) receptor. This receptor binds low density lipoproteins (LDL) poorly and is more resistant to cholesterol down-regulation than the receptor on human fibroblasts. The properties of the apoB,E(LDL) receptor on Fu5AH cells are similar to those found on mouse peritoneal macrophages (12) and distinct from those of the apo B,E(LDL) receptor on rat fibroblasts and smooth muscle cells (13).

MATERIALS AND METHODS

Lipoproteins

Canine plasma lipoproteins were obtained from dogs that had been fasted overnight. Canine LDL were obtained from the plasma of foxhounds fed a commercial dog chow. The cholesterol-induced high density lipoproteins containing apoE (apoE HDL_c) and the β-VLDL were prepared from plasma obtained from foxhounds fed a semi-synthetic diet containing cholesterol and hydrogenated coconut oil (14). The LDL from control dogs were isolated by ultracentrifugation (d 1.02–1.063 g/ml) in a 60 Ti rotor (Beckman Instruments, Mountain View, CA) at 59,000 rpm for 18 hr and were purified by Pevikon block electrophoresis (Pevikon Corp., Mercer Consolidated Corp., Yonkers, NY) (15). The LDL isolated by this procedure contained only apoB-100. Apolipoprotein E was not detectable in the LDL fraction on SDS-polyacrylamide gels by either Coomassie blue staining or silver staining methods. The β-VLDL were isolated from the d < 1.006 g/ml fraction by Pevikon block electrophoresis, and the apoE HDL_c from the d 1.006–1.02 g/ml fraction, as described (14). Ultracentrifugation (d 1.006–1.063 g/ml) and Pevikon block electrophoresis (13) were used to isolate rat LDL from plasma of normal Sprague-Dawley rats (200–300 g) that had been maintained on commercial laboratory chow. Chylomicron remnants were obtained from chylomicrons isolated from dog lymph after incubation with human postheparin lipolytic plasma, as described (16). The isolated lipoproteins were dialyzed extensively against 0.15 M NaCl, 0.01% ethylenediaminetetraacetate (EDTA), pH 7.4, before use. Chylomicron remnants, LDL, and β-VLDL were radiolabeled with ^{125}I by the iodine monochloride method (17). The apoE HDL_c were iodinated by the Bolton-Hunter procedure (18). Reductively methylated canine ^{125}I-labeled β-VLDL were prepared by treatment of the lipoprotein with formaldehyde plus sodium borohydride, as described (19).

Protein concentrations were determined by the method of Lowry et al. (20), using bovine serum albumin as a protein standard. Cholesterol and triglyceride concentrations were determined by enzymatic methods (Bio-Dynamics, Boehringer-Mannheim, Indianapolis, IN), and phospholipid was measured by an assay for phosphorus content (21). After delipidation, the apolipoproteins of the lipoproteins were characterized by gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) on 5–20% polyacrylamide gradient gels (22). The gels were stained with silver nitrate to identify the protein bands (23).

Preparation of membranes

Membranes from rat liver and dog adrenal were prepared according to the procedure of Kovanen et al. (24). Membranes from Fu5AH cells were prepared from cells grown to confluency in 100-mm dishes. Cells on the plates were chilled, washed twice with phosphate-buffered saline (PBS), and scraped into PBS supplemented with 0.5 mM phenylmethylsulfonyl fluoride (PMSF), Trasylol 1 kIU/ml, and 0.1 mM leupeptin. All subsequent steps were performed at 4°C. The cells were collected by centrifugation, resuspended in Tris-buffered saline containing 1 mM $CaCl_2$, 0.5 mM PMSF, and 0.1 mM leupeptin, and subjected to homogenization with a Polytron (two 10-sec bursts on setting No. 8 at a 1-min interval). Nuclei and cell debris were sedimented from the homogenate by centrifugation for 10 min at 800 *g*, and the membrane fraction was collected by centrifugation at 100,000 *g* for 60 min. Membranes were resuspended in a small volume of Tris-buffered saline containing Trasylol 1 kIU/ml, leupeptin 0.1 mM, and PMSF 0.5 mM, and were dispersed by brief sonication (Branson Sonifier, 10 sec, setting No. 6, using the microprobe). Membrane binding assays were performed at 4°C in 100 μl of incubation buffer containing 50 mM Tris-HCl (pH 7.5), 25 mM NaCl, 1.0 mM $CaCl_2$, and 20 mg of bovine serum albumin/ml. Each in-

cubation was performed with 100 μg of membrane protein, as described by Basu, Goldstein, and Brown (25). Nonspecific calcium-independent binding, determined by inclusion of 20 mM EDTA in the assay tube, was 50% of total binding for LDL and was 40–50% of total binding observed for apoE HDL_c and β-VLDL.

Preparation of cell extracts

Triton extracts were prepared from Fu5AH cells or cultured human fibroblasts grown to confluency in 60-mm dishes. Cells were chilled, then washed twice with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline containing 2 mM $CaCl_2$. To each plate was added 0.1 ml of solubilization buffer (10 mM HEPES, pH 7.4, 2.5 mM $MgCl_2$, 200 mM NaCl, 2 mM $CaCl_2$, 0.5 mM PMSF, 0.1 mM leupeptin, and 1.5% (v/v) Triton X-100). The solubilized cells were scraped from dishes, pooled, and centrifuged at 130,000 *g* for 30 min at 4°C (Beckman airfuge). Supernatant fractions were pooled and stored at −20°C. Protein contents of the Triton extracts were determined by Lowry assays of trichloroacetic acid–deoxycholate precipitates (26).

Cell culture and assay

The Fu5AH cells and cultured human fibroblasts were maintained as previously described (3, 4, 17, 18). Stock cultures of Fu5AH cells were grown in minimal essential medium (MEM) supplemented with basal Eagle's medium, 100 μg of penicillin/ml, 100 μg of streptomycin/ml (all from GIBCO, Grand Island, NY), and 5% calf serum (HyClone, Logan, UT). These were maintained at 37°C in a humidified incubator at 5–8% CO_2. Cells from stock culture were detached using 0.05% trypsin and 0.02% EDTA and plated in 16-mm petri dishes at a density of 3 to 4×10^4 cells per dish. After 4 days in culture, the cells were washed once with MEM containing 5% lipoprotein-deficient human serum, followed by the addition of 1 ml of medium containing 10% lipoprotein-deficient human serum. All experiments were performed on day 6. Cultures of normal rat fibroblasts were grown as described (13). Assays for binding and degradation of lipoproteins and for lipoprotein-induced cholesterol esterification were performed as described (17, 18). Nonspecific binding and degradation were determined with a 100-fold excess of unlabeled ligand and were less than 20% of the total binding or degradation for each lipoprotein. Details of individual experiments are described in the figure legends. Unless otherwise indicated, each figure displays a single typical experiment and each point represents the average of duplicate determinations.

Antibodies

A polyclonal antibody raised against the purified bovine adrenal apoB,E(LDL) receptor was prepared as described (27, 28). An anti-apoE monoclonal antibody (1D7) was provided by Drs. Yves Marcel and Ross Milne, Clinical Research Institute of Montreal, Canada (29). Anti-peptide antibodies against the extreme carboxyl-terminal 14 amino acids of the apoB,E(LDL) receptor were raised in rabbits by using a procedure similar to that described by Schneider et al. (30). These antibodies react with the human and rat apoB,E(LDL) receptor (30).

Ligand blotting and immunoblotting

One-dimensional electrophoresis was carried out on 5–20% polyacrylamide slab gels containing 0.1% (v/v) SDS as described (22). The samples were adjusted to a final concentration of 62.5 mM Tris-HCl, 3% SDS, 0.1% bromphenol blue, and 10% (v/v) glycerol, and incubated 1 hr at 37°C before being loaded onto the gel (no reducing agent was used). Proteins were transferred from SDS-slab gels to nitrocellulose paper electrophoretically, according to the method of Beisiegel et al. (31). The nitrocellulose paper was incubated with either lipoproteins or antibodies, as described (32). Autoradiograms were obtained by exposing the dried paper with enhancing screens to Kodak XAR-5 film for 24 to 48 hr.

RESULTS

Previous studies have shown that incubation of Fu5AH rat hepatoma cells with hypercholesterolemic $d < 1.006$ g/ml lipoproteins (β-VLDL) resulted in a massive accumulation of cellular cholesteryl ester (1–3). To determine whether this cholesteryl ester accumulation was the result of the uptake of β-VLDL by lipoprotein receptors, the cellular uptake and degradation of the ^{125}I-labeled β-VLDL from cholesterol-fed dogs were examined at 37°C. As shown in **Fig. 1**, canine ^{125}I-labeled β-VLDL were taken up and degraded by a specific, high-affinity, saturable process. Furthermore, the high-affinity binding, uptake, and degradation by Fu5AH cells were abolished by selective chemical modification of lysyl residues of the apolipoproteins by reductive methylation, a procedure previously shown to prevent apoB- or E-containing lipoproteins from binding to lipoprotein receptors (19) (Fig. 1). Therefore, the high-affinity uptake and degradation of canine β-VLDL by Fu5AH cells are dependent on the binding of β-VLDL to specific cell-surface receptors.

One of the consequences of the receptor-mediated uptake of cholesterol-rich lipoproteins is the stimulation of cellular cholesteryl ester formation. When Fu5AH cells were incubated for 5 hr with canine β-VLDL, there was a marked stimulation of cholesteryl ester formation, as evidenced by the increased rate of incorporation of [^{14}C]oleate into cellular cholesteryl esters **(Fig. 2)**. Reductive methylation of the lysine residues in β-VLDL apoli-

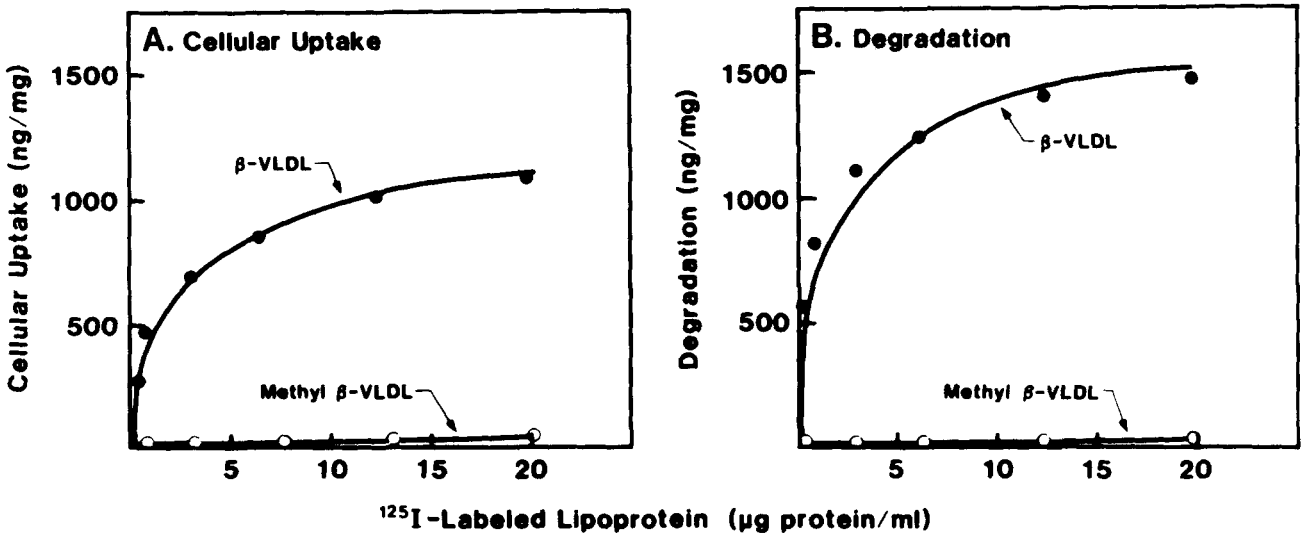

Fig. 1. Comparison of the cellular uptake (A) and degradation (B) of canine ^{125}I-labeled β-VLDL (●) and canine ^{125}I-labeled, reductively methylated β-VLDL (○) by Fu5AH rat hepatoma cells as a function of the concentration of the lipoproteins in the medium. Each dish received 0.5 ml of minimal essential medium (MEM) containing 10% lipoprotein-deficient human serum and the indicated concentration of ^{125}I-labeled β-VLDL (875 cpm/ng of protein) or ^{125}I-labeled, reductively methylated β-VLDL (801 cpm/ng of protein). After incubation for 5 hr at 37°C, the amounts of (A) ^{125}I in the cells and (B) ^{125}I-labeled acid-soluble material in the medium were determined.

poproteins profoundly diminished the rate of cholesteryl ester synthesis. These results indicate that most of the cholesteryl ester that accumulates in Fu5AH cells after incubation with β-VLDL is the result of uptake of the lipoproteins by a receptor-mediated process.

The studies of Pattnaik and Zilversmit (9) suggested that the C apolipoproteins on chylomicron remnants mediated the uptake of these lipoprotein particles by Fu5AH cells. However, the high-affinity binding of canine ^{125}I-labeled β-VLDL to cell-surface receptors on Fu5AH cells suggested that the interaction of β-VLDL with these cells was mediated by apoE. To test this hypothesis, lipoproteins were preincubated with the anti-apoE monoclonal antibody 1D7 before the binding assays were performed. This antibody was shown previously to inhibit binding of apoE-containing lipoproteins to lipoprotein receptors on mammalian cells (29). Results showed that the 1D7 antibody (20 μg/ml) inhibited the binding of canine ^{125}I-labeled apoE HDL$_c$ (90%), ^{125}I-labeled β-VLDL (80%), and ^{125}I-labeled chylomicron remnants (100%) to the Fu5AH cells (data not shown). Thus, apoE is the primary determinant for mediating the interaction of β-VLDL and chylomicron remnants with the cell-surface receptors of Fu5AH rat hepatoma cells.

To characterize the lipoprotein receptors on Fu5AH cells in more detail, direct binding studies were performed at 4°C. Direct binding of canine ^{125}I-labeled β-VLDL, ^{125}I-labeled LDL, ^{125}I-labeled apoE HDL$_c$, and ^{125}I-labeled chylomicron remnants occurred as a function of lipoprotein concentration in the medium **(Fig. 3)**. All of these lipoproteins demonstrated saturable binding to the Fu5AH cells. The affinity of these lipoproteins for the receptor and the maximum amount of lipoprotein bound to the receptors at receptor saturation were calculated by the method of Scatchard (33). As shown in **Fig. 4** and summarized in **Table 1**, the apoE-containing lipoproteins (^{125}I-labeled apoE HDL$_c$, ^{125}I-labeled chylomicron remnants, and ^{125}I-labeled β-VLDL) displayed a much higher binding affinity to the Fu5AH cells than did the canine ^{125}I-labeled LDL. Whereas the equilibrium dissociation constants (K_d) for apoE-containing lipoproteins were < 1 μg/ml, the K_d for canine LDL was 22 μg/ml (24×10^{-9}M). Rat LDL bound to the Fu5AH cell-surface receptors with

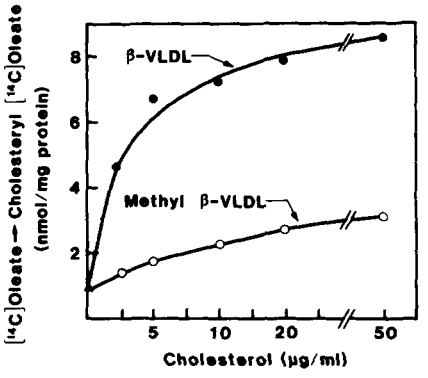

Fig. 2. Comparison of the ability of canine β-VLDL (●) and reductively methylated canine β-VLDL (○) to stimulate cholesteryl ester formation in Fu5AH rat hepatoma cells. Each dish received 0.5 ml of MEM containing 0.2 mM [^{14}C]oleate-albumin, and the indicated concentration of lipoprotein cholesterol. After incubation for 5 hr at 37°C, the cellular content of cholesteryl [^{14}C]oleate was determined.

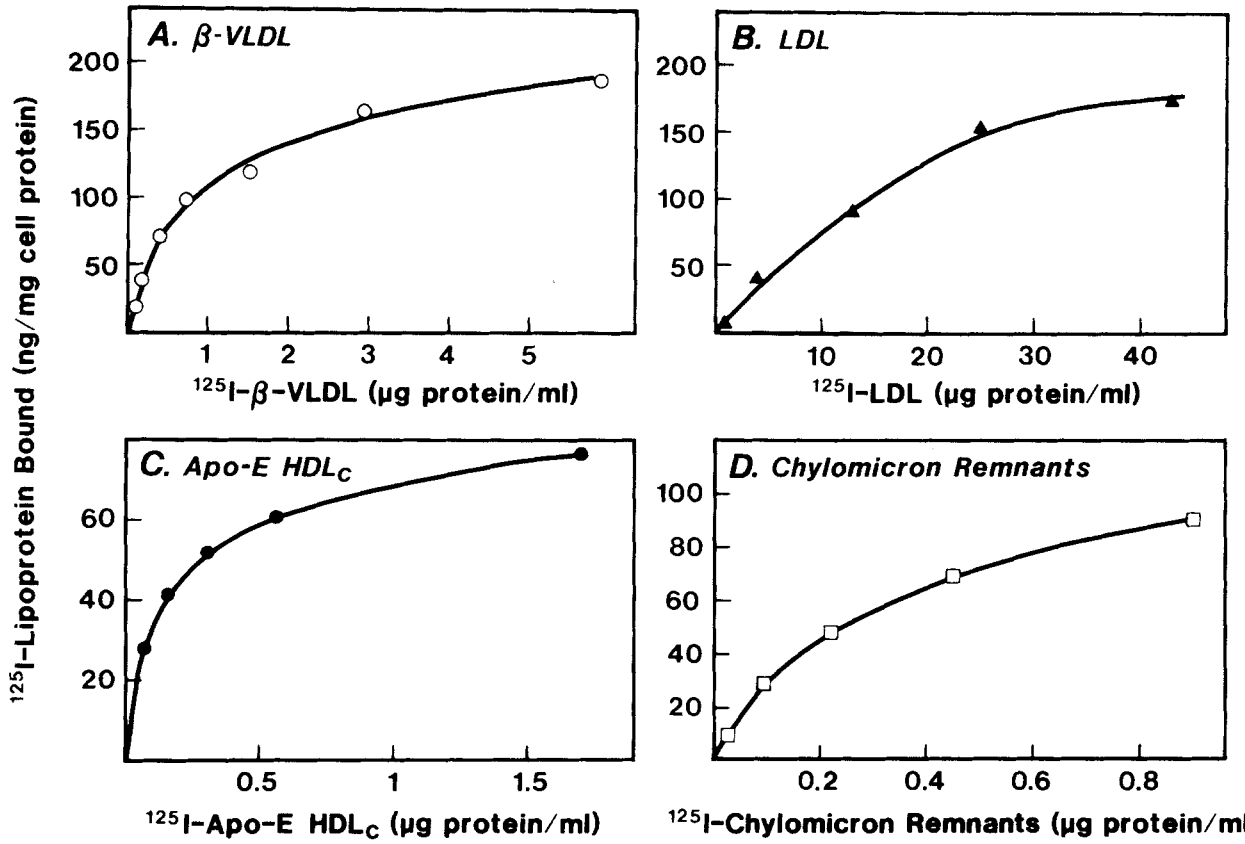

Fig. 3. Concentration-dependent binding of (A) canine ^{125}I-labeled β-VLDL (831 cpm/ng of protein), (B) canine ^{125}I-labeled LDL (420 cpm/ng of protein), (C) canine ^{125}I-labeled apoE HDL$_c$ (462 cpm/ng of protein), or (D) ^{125}I-labeled chylomicron remnants (600 cpm/ng of protein) to Fu5AH rat hepatoma cells. Each dish was incubated for 3 hr at 4°C with 0.5 ml of MEM containing 10% lipoprotein-deficient human serum and the iodinated lipoproteins at the concentrations indicated. Data have been corrected for nonspecific binding. Each point represents the average of duplicate determinations.

a similarly low affinity (K_d = 20 μg/ml; 22 × 10^{-9} M) **(Fig. 5** and Table 1). By comparison, rat and human LDL bind to the apoB,E(LDL) receptors on human skin fibroblast with a K_d of 1.8 μg/ml, or 2.8 × 10^{-9}M (34). The affinity of apoE-containing lipoproteins that bind to Fu5AH is similar to that of apoE-containing lipoproteins that bind to human fibroblasts (data not shown). However, the affinity of LDL binding to Fu5AH cells is approximately 5- to 10-fold less than that observed for many other cell lines, including rat fibroblasts and rat smooth muscle cells (13, 34). These data indicate that Fu5AH cells possess receptors with a high affinity for apoE but with a substantially reduced affinity for apoB. The unexpected very low-affinity binding of LDL to the cell-surface receptors on Fu5AH cells was confirmed by membrane binding assays. **Table 2** compares the binding of canine LDL and apoE HDL$_c$ to isolated rat liver membranes and Fu5AH cell membranes. The very low binding affinity of LDL to Fu5AH cell-surface receptors was observed when the Ca^{2+}-dependent binding of the lipoprotein was determined in membranes prepared from Fu5AH cells.

Furthermore, data in Table 2 indicate the presence of apoE receptors in rat liver membranes and the absence of functional apoE receptors on Fu5AH cells. This conclusion is based on the following findings from previous studies: first, the apoB,E(LDL) receptor binds both apoB- and E-containing lipoproteins, whereas the apoE receptor binds only apoE-containing lipoproteins; and second, the apoB,E(LDL) receptor binds three- to fourfold more LDL than apoE HDL$_c$ (34). Cultured rat fibroblasts, which are known to possess the apoB,E(LDL) receptor, exhibit this ratio of LDL to apoE HDL$_c$ binding (Table 2). However, rat liver membranes display nearly a 1:1 binding ratio, suggesting that both apoB,E(LDL) and apoE receptors are present. This is consistent with results obtained with liver membranes from other species (10, 11). By contrast to the rat liver membranes, the Fu5AH membranes have a 4:1 ratio of LDL to apoE HDL$_c$ binding, suggesting the presence of only the apoB,E(LDL) receptor (Table 2). Likewise, the cultured Fu5AH cells displayed a 3.5:1 ratio of LDL to apoE HDL$_c$ binding, another indicator of the presence of the apoB,E(LDL) receptor.

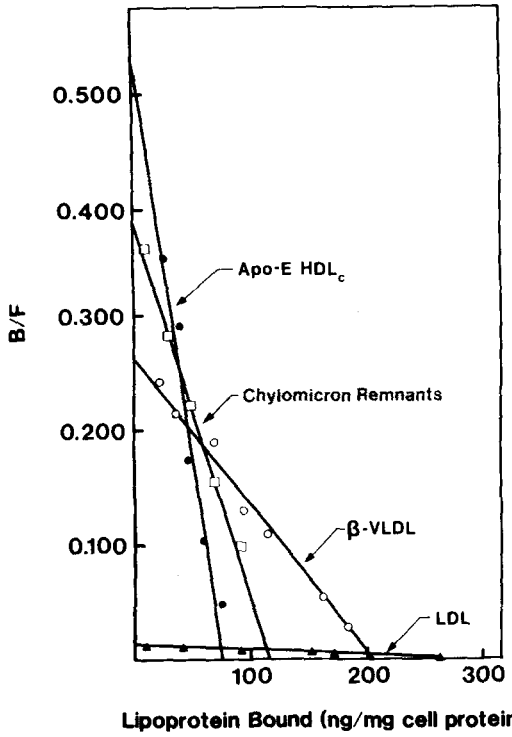

Fig. 4. Scatchard plot for binding of apoE HDL$_c$ (●), chylomicron remnants with apoE (□), canine β-VLDL (○), and canine LDL (▲) to Fu5AH rat hepatoma cells. Bound/free (B/F) refers to the amount of lipoprotein bound (ng of protein/mg of cell protein)/amount of lipoprotein free in the medium (ng of protein/ml). Data derived from Fig. 3.

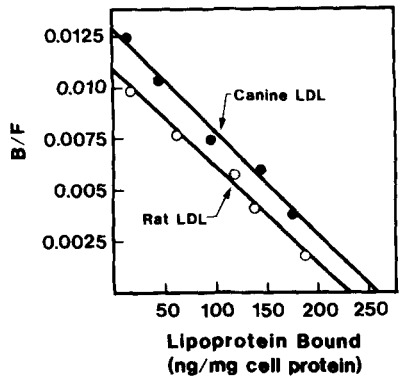

Fig. 5. Scatchard plot for binding of rat ^{125}I-labeled LDL (○) and canine ^{125}I-labeled LDL (●) to Fu5AH rat hepatoma cells. Binding assays were performed at 4°C as in Fig. 3. The specific activity of rat LDL and canine LDL was 510 and 420 cpm/ng of protein, respectively. The maximum amount of rat and canine LDL bound was 229 and 253 ng/mg of cell protein, respectively.

The lack of a functional apoE receptor and the presence of the apoB,E(LDL) receptor on Fu5AH cells were confirmed by competitive binding studies. The ability of unlabeled canine LDL to compete effectively with canine ^{125}I-labeled β-VLDL, ^{125}I-labeled apoE HDL$_c$, and ^{125}I-labeled chylomicron remnants for receptor binding is shown in **Fig. 6**. High concentrations of LDL were required; this reflected the low affinity of LDL for the apoB,E(LDL) receptor. Because LDL do not displace ^{125}I-labeled β-VLDL, ^{125}I-labeled apoE HDL$_c$, or ^{125}I-labeled chylomicron remnants from the apoE receptor, these results also indicate the lack of a functional apoE receptor on these cells.

Consideration was given to the possibility that the massive accumulation of cholesteryl esters by Fu5AH cells, as compared with fibroblasts, was due to a lack of effective down-regulation of their apoB,E(LDL) receptors. Monolayers of Fu5AH and human fibroblasts were incubated with increasing concentrations of canine β-VLDL in 10% lipoprotein-deficient human serum for 48

TABLE 1. Equilibrium dissociation constants (K_d) for various lipoproteins binding to different cell types in culture

Cells in Culture	Equilibrium Dissociation Constant[a]					
	Rat LDL	Canine LDL	Human LDL	Canine ApoE HDL$_c$	Canine β-VLDL	Canine Chylomicron Remnants
Fu5AH[b]	20 (22)	22 (24)	–	0.14 (0.26)	0.96 (N.D.)[c]	0.3 (0.01)
Rat fibroblasts	2.5 (2.8)	5.7 (6.3)	–	0.13 (0.24)	–	–
Rat smooth muscle cells	5.2[d] (5.7)	–	–	–	–	–
Human fibroblasts	–	–	1.8[d] (2.8)	0.065[d] (0.12)	–	–

[a] The equilibrium dissociation constants are expressed in two ways. Because the molecular weights of the lipoproteins are only approximate, the K_d values are first given in units taken directly from the experiments (μg of proteins/ml). The K_d values in parentheses are nanomolar values ($\times 10^{-9}$ M). The following molecular weights and protein composition percentages were used for computation: human LDL, M_r 3.0 $\times 10^6$, 21% protein; canine apoE HDL$_c$, M_r 3.6 $\times 10^6$, 15% protein; rat and canine LDL, M_r 3.0 $\times 10^6$, 30% protein; rat chylomicron remnants, M_r 218.0 $\times 10^6$, 13% protein.

[b] Each point is an average of two experiments.

[c] N.D., not determined.

[d] From Refs. 13 and 34.

TABLE 2. Binding of canine LDL and apoE HDL_c to cultured rat fibroblasts, Fu5AH cells, rat liver membranes, and Fu5AH cell membranes

	Canine LDL		ApoE HDL_c	
	K_d (M)	B_{max} (ng/mg protein)	K_d (M)	B_{max} (ng/mg protein)
A. Cells in culture				
Rat fibroblasts	6.3×10^{-9}	199	2.4×10^{-10}	52
Rat hepatoma (Fu5AH)	3.2×10^{-8}	168	2.6×10^{-10}	48
B. Membranes				
Rat liver	9.0×10^{-9}	27	7.4×10^{-10}	40
Rat hepatoma (Fu5AH)	2.9×10^{-8}	144	8.5×10^{-10}	35

hr at 37°C. At the end of this incubation, the cells were washed and the level of β-VLDL binding at 4°C was determined. As demonstrated in **Fig. 7**, the binding of ^{125}I-labeled β-VLDL was greatly reduced by prior incubation of both cell types with β-VLDL. However, the dose dependence of receptor down-regulation in Fu5AH cells was decisively different from that of human fibroblasts (Fig. 7). Whereas a 50% reduction of receptors was achieved with only 3 μg of β-VLDL cholesterol/ml for the human fibroblasts, Fu5AH cells required 20 μg of β-VLDL cholesterol/ml for 50% down-regulation, and complete down-regulation was observed only with 500 μg of β-VLDL cholesterol/ml.

To characterize the apoB,E(LDL) receptor on Fu5AH cells in more detail, a rabbit polyclonal antibody raised against purified bovine adrenal apoB,E(LDL) receptor was used. This antibody cross-reacts with apoB,E(LDL) receptors of several species and inhibits lipoprotein binding to cultured human fibroblasts and mouse peritoneal macrophages (12, 28). When Fu5AH cells were preincubated with the apoB,E(LDL) receptor antibody, the binding of canine ^{125}I-labeled LDL, ^{125}I-labeled β-VLDL, and ^{125}I-labeled apoE HDL_c to Fu5AH cell-surface receptors was inhibited **(Fig. 8)**. Thus, the Fu5AH cell-surface receptors are immunologically similar to apo B,E(LDL) receptors.

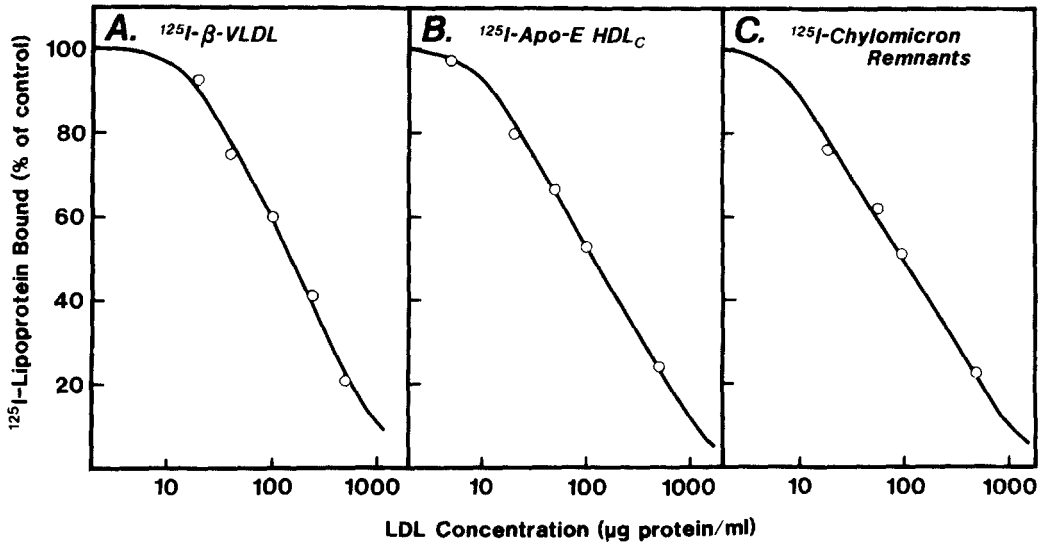

Fig. 6. The ability of unlabeled canine LDL to compete with (A) canine ^{125}I-labeled β-VLDL, (B) canine ^{125}I-labeled HDL_c, and (C) canine ^{125}I-labeled chylomicron remnants for binding to Fu5AH rat hepatoma cells. Each dish received 0.3 ml of MEM containing 10% lipoprotein-deficient human serum. Canine ^{125}I-labeled β-VLDL (0.1 μg of protein/ml), ^{125}I-labeled apoE HDL_c (0.1 μg of protein/ml), or canine ^{125}I-labeled chylomicron remnants (0.1 μg of protein/ml) were added to the incubation medium along with unlabeled canine LDL. After incubation for 2 hr at 4°C, the cells were washed extensively with cold phosphate-buffered saline containing 2 mg/ml of bovine serum albumin and then solubilized in 0.1 M NaOH prior to counting radioactivity. The 100% control values for the β-VLDL, apoE HDL_c, and chylomicron remnants bound were 29, 44, and 56 ng/mg of cellular protein, respectively.

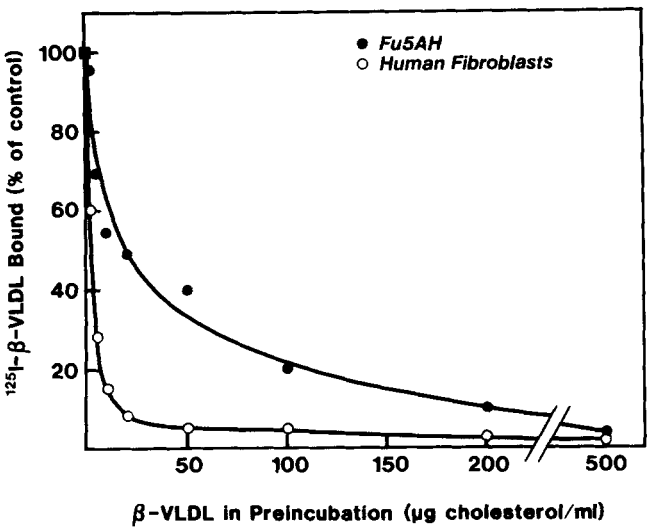

Fig. 7. Comparison between the down-regulation of the expression of apoB,E(LDL) receptors on Fu5AH cells and on cultured human fibroblasts after prior incubation of the cells with β-VLDL. After cell monolayers in 22-mm wells were washed with serum-free medium, 1.5 ml of medium containing 10% lipoprotein-deficient human serum and the indicated amount of β-VLDL cholesterol was added to each well. After 48 hr at 37°C, the medium was aspirated and cells were incubated for 1 hr at 37°C in Dulbecco's modified essential medium–10% lipoprotein-deficient human serum to allow for internalization of surface-bound lipoproteins prior to the binding experiments. Cells were then chilled and the binding of 1 μg of canine ^{125}I-labeled β-VLDL/ml was measured at 4°C as in Fig 3. All data are corrected for nonspecific binding. Data shown are the average of five experiments for Fu5AH cells and ten experiments for human fibroblasts.

The molecular weight of the Fu5AH receptor was determined on immunoblots by using the apoB,E(LDL) receptor antibody. As shown on nonreduced gels in **Fig. 9**, the apoB,E(LDL) receptor of dog adrenal and rat liver membranes had a $M_r \simeq 130{,}000$. By comparison, the Fu5AH cell membranes possessed two bands: $M_r \simeq 130{,}000$ and 115,000. Upon reduction with dithiothreitol, the $M_r \simeq 130{,}000$ and 115,000 bands displayed an apparent $M_r \simeq 160{,}000$ and 145,000 (retaining the $M_r \simeq 15{,}000$ difference) (data not shown). Previously, Schneider, Goldstein, and Brown (26) demonstrated that the apoB,E(LDL) receptor of bovine adrenals had a $M_r \simeq 120{,}000$ (nonreduced) and 160,000 (reduced). Furthermore, as shown in **Fig. 9**, ligand blotting with ^{125}I-labeled apoE HDL$_c$ revealed calcium-dependent binding to both bands (the binding was abolished by EDTA treatment). Treatment of the Fu5AH cells in culture with pronase prior to membrane isolation resulted in the disappearance of both bands from subsequent immunoblots, demonstrating that both the $M_r \simeq 130{,}000$ and 115,000 proteins were pronase-sensitive in the intact cells and thus were oriented externally on the cell surface (data not shown).

The lower molecular weight form of the Fu5AH receptor ($M_r \simeq 115{,}000$) was absent from Triton extracts of the cells as compared with the isolated cell membranes. As shown in **Fig. 10**, the solubilized cells contained only the $M_r \simeq 130{,}000$ protein, as detected by apoB,E(LDL) receptor antibodies or apoE HDL$_c$ ligand blotting. However, the $M_r \simeq 115{,}000$ protein was present in the membrane fraction. To determine whether the 115-kDa protein was derived from the 130-kDa receptor during processing of the cells for membrane isolation, the Fu5AH cells were either extracted with Triton directly or disrupted by Polytron sonication and then extracted with Triton. A single band of apoE HDL$_c$ binding activity at $M_r \simeq 130{,}000$ protein was observed in cells solubilized directly with Triton without homogenization (**Fig. 11**, Control), even in the absence of antiproteolytic agents. However, Triton extracts of Polytron-disrupted Fu5AH cells contained both the $M_r \simeq 115{,}000$ and 130,000 proteins (Fig. 11, Total). Densitometric scans of the apoE HDL$_c$ ligand blots revealed that in the total homogenate, 36% of ligand binding occurred with the $M_r \simeq 130{,}000$ and 64% with the $M_r \simeq 115{,}000$ protein. Before Triton extraction, a portion of the Fu5AH cell homogenate was centrifuged at 130,000 *g*, which resulted in a pellet containing the membranes and a supernatant fraction. Only the $M_r \simeq 115{,}000$ protein was present in the supernatant fraction (Fig. 11, Super). In the pellet, roughly equal amounts of the $M_r \simeq 130{,}000$ and 115,000 forms of the receptor were found. These results are consistent with the $M_r \simeq 115{,}000$ protein representing a cleavage product of

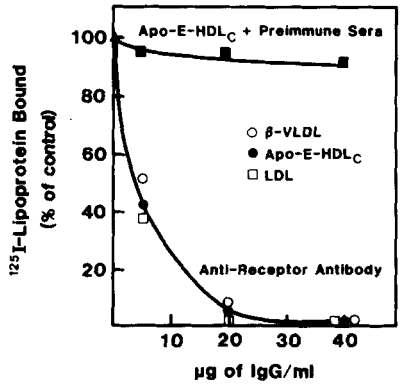

Fig. 8. Inhibition of binding of canine ^{125}I-labeled LDL, canine ^{125}I-labeled β-VLDL, and ^{125}I-labeled apoE HDL$_c$ to rat Fu5AH cells by polyclonal anti-apoB,E(LDL) receptor antibodies. Each dish received 0.5 ml of ice-cold MEM containing 10% lipoprotein-deficient human serum and the indicated concentration of polyclonal anti-apoB,E(LDL) receptor antibodies. After incubation for 1.5 hr at 4°C, canine ^{125}I-labeled LDL (5.2 μg/ml, 247 cpm/ng of protein), ^{125}I-labeled β-VLDL (0.1 μg/ml, 332 cpm/ng of protein), and ^{125}I-labeled apoE HDL$_c$ (0.1 μg/ml, 338 cpm/ng of protein) were added to give the final concentration indicated. The cells were incubated for an additional 1.5 hr at 4°C, after which the specific amount of the labeled lipoproteins bound to the cells was determined. The 100% values for canine LDL, canine β-VLDL, and apoE HDL$_c$ were 69, 33, and 40 ng of protein bound/mg of cell protein, respectively. IgG, immunoglobulin G.

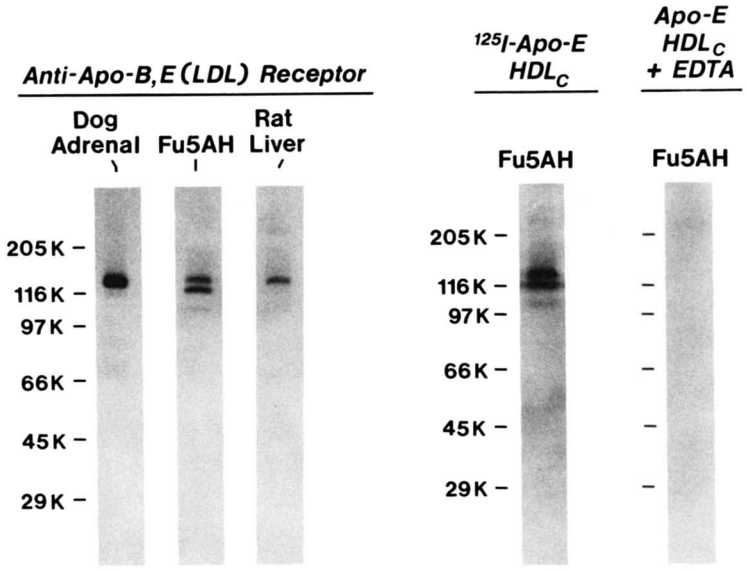

Fig. 9. Western blots of apoB,E(LDL) receptors from isolated membranes of canine adrenal, Fu5AH rat hepatoma cells, and rat liver. Isolated membranes were prepared as described under Materials and Methods, and were not solubilized prior to gel electrophoresis but were mixed with the sodium dodecyl sulfate (SDS) application buffer directly. The electrophoresis was performed on 5–20% polyacrylamide gradient gels containing 0.1% SDS, followed by electrophoretic transfer to nitrocellulose. Relative migration of molecular weight standards is indicated for each set of blots. Left: Immunoblots of canine adrenal, Fu5AH cell, and rat liver membranes are shown. Samples containing 250 μg of protein were incubated in sample application buffer in the absence of reducing agents for 60 min at 37°C prior to electrophoresis. The nitrocellulose paper was incubated with 50 μg/ml of a polyclonal antibody raised against purified apoB,E(LDL) receptor from bovine adrenal. Following incubation, the blot was incubated with ^{125}I-labeled donkey anti-rabbit immunoglobulin G (2.5×10^5 cpm/ml). The dried papers were processed for autoradiography. Right: Ligand blots of isolated membranes from Fu5AH cells are shown. The blot was incubated with 1 μg of protein/ml of ^{125}I-labeled apoE HDL_c (600 cpm/ng) in the absence or presence of 20 mM EDTA, then washed, dried, and processed for autoradiography.

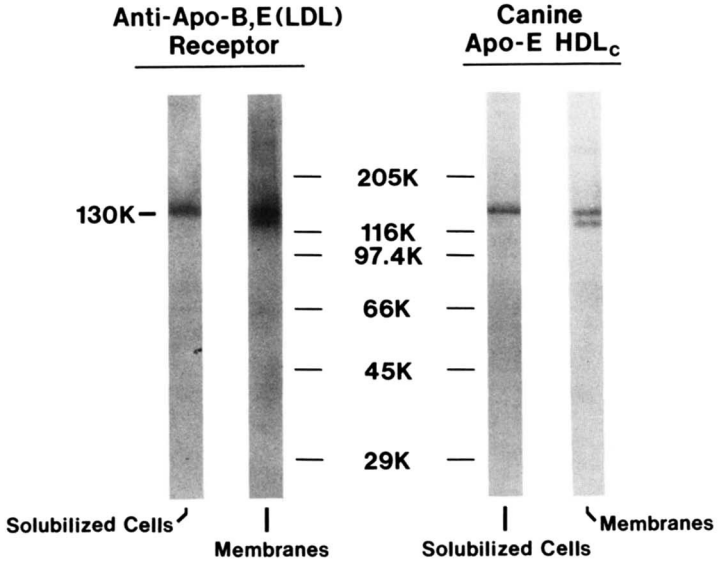

Fig. 10. Western blots of apoB,E(LDL) receptors from isolated membranes and Triton extracts of Fu5AH cells. Samples containing 220 μg of protein from Triton-solubilized Fu5AH cells or from isolated membranes of Fu5AH cells were dissolved in application buffer, subjected to electrophoresis in 5–20% slab gels containing 0.1% sodium dodecyl sulfate (SDS), and transferred electrophoretically to nitrocellulose paper. Left: Immunoblot; the nitrocellulose was incubated with 50 μg of apoB,E(LDL) receptor antibody/ml followed by incubation with ^{125}I-labeled donkey anti-rabbit immunoglobulin G and was processed for autoradiography. Right: Ligand blot; the blot was incubated with 1 μg of ^{125}I-labeled apoE HDL_c (600 cpm/ng)/ml, washed, dried, and processed for autoradiography. Relative migrations of molecular weight standards are indicated.

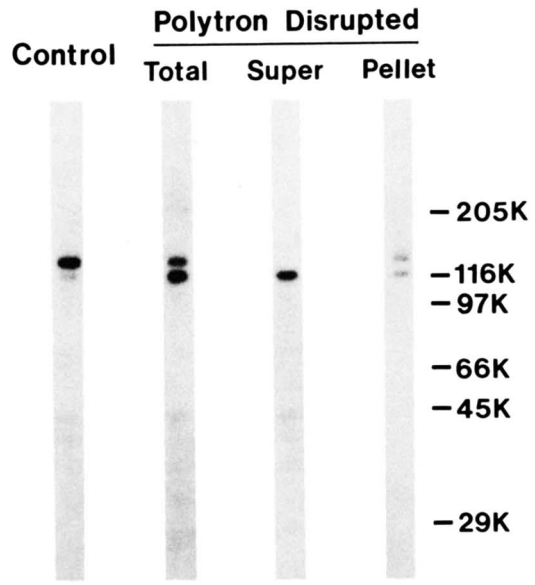

Fig. 11. Production and release of a 115-kDa fragment of the apoB,E(LDL) receptor in Polytron-disrupted Fu5AH cells. The control extract was prepared by solubilization of Fu5AH cells with Triton X-100 as described in Materials and Methods. A homogenate of Fu5AH cells was prepared by scraping 40 confluent 60-mm dishes of cells into 4 ml of solubilization buffer from which Triton X-100 was omitted, then subjecting the cell suspension to Polytron disruption for 10 sec. A portion of the homogenate was brought immediately to 1.5% (v/v) Triton X-100 by addition of a 30% (v/v) Triton X-100 stock. The remainder of the homogenate was centrifuged at 130,000 *g*. The pellets were resuspended in HEPES-buffered saline equivalent to the original volume, and Triton extracts were prepared from aliquots of the supernatant and pellet fractions. The Triton extracts of each sample were analyzed by polyacrylamide gel electrophoresis and apoE HDL_C ligand blot binding; (Control) cells solubilized into Triton directly from plates, 175 μg of protein; (Total) Triton extract of Fu5AH cell homogenate, 175 μg of protein; (Super) Triton-treated 130,000 *g* supernate of homogenate from an equivalent volume of homogenate; and (Pellet) Triton extract of 130,000 *g* pellet from equivalent volume of homogenate.

the $M_r \simeq 130{,}000$ protein. Some of the lower molecular weight form is centrifuged off the membrane, while the remainder is trapped or loosely adherent to the membrane.

Further characterization of the $M_r \simeq 115{,}000$ protein revealed that it lacked the carboxyl-terminal amino acids. A rabbit polyclonal antibody, prepared against a synthetic peptide comprising the extreme carboxyl-terminal 14 amino acids of the human apoB,E(LDL) receptor, reacted against the $M_r \simeq 130{,}000$ protein from Fu5AH cells but not against the $M_r \simeq 115{,}000$ fragment in the supernatant fraction **(Fig. 12)**. Based on the structure and the orientation of the apoB,E(LDL) receptor in the plasma membrane (35, 36), approximately 90% of the receptor is on the cell surface, including the postulated ligand binding domain that has been localized to the amino terminus of the receptor. Adjacent to the plasma membrane on the external surface of the cell is a region containing *O*-glycosylation sites. The membrane-spanning region of the receptor, composed of 22 hydrophobic amino acids, is connected to the carboxyl-terminal 50 amino acids that make up the cytoplasmic domain in the interior of the cell. Therefore, the absence of reactivity with a carboxyl-terminal antibody, in the face of ligand binding, is consistent with the $M_r \simeq 115{,}000$ protein representing the amino-terminal 90% of the apoB,E(LDL) receptor.

DISCUSSION

The rat-derived Fu5AH hepatoma cells are an interesting model system for studying cellular cholesterol metabolism because of their ability to accumulate large cholesteryl ester droplets during incubation with hyperlipidemic serum or with the d < 1.006 g/ml fraction (β-VLDL) from hyperlipidemic serum (1–3). This report demonstrates that β-VLDL uptake by Fu5AH cells is receptor-mediated: the metabolism of β-VLDL is high-affinity, saturable, and subject to inhibition by chemical modification of the lysyl residues of the apolipoproteins. In a manner analogous to the interaction of β-VLDL with previously examined receptors on human fibroblasts, canine liver membranes, and mouse peritoneal macro-

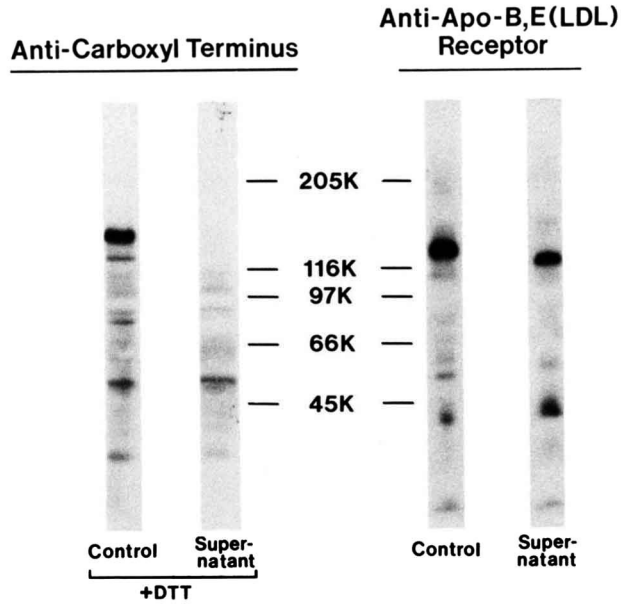

Fig. 12. Inability of an antibody against the carboxyl terminus of the apoB,E(LDL) receptor to recognize the receptor fragment released from Fu5AH cells. Triton extracts of Fu5AH cell monolayers (Control) and of the supernatant fraction of Fu5AH cell homogenate (Supernatant) were prepared as in Fig. 11. Left: Samples containing control extract (265 μg protein) or supernatant (equivalent 190 μg of protein volume) were incubated in sample buffer with 100 μM dithiothreitol for 60 min at 37°C, then electrophoresed and transferred to nitrocellulose paper as described. The blot was incubated with 50 μg/ml of an antibody against the carboxyl-terminal region of the apoB,E(LDL) receptor followed by incubation with ^{125}I-labeled donkey anti-rabbit immunoglobulin G. Right: Samples were incubated with sample buffer in the absence of reducing agents at 37°C for 60 min. The blot was reacted with anti-apoB,E(LDL) receptor antibody and donkey anti-rabbit immunoglobulin G as in Fig. 9.

phages (12, 16, 37), apoE is the determinant of β-VLDL binding to the receptors of Fu5AH cells. The only functional lipoprotein receptors found on Fu5AH cells, however, are apoB,E(LDL) receptors; the Fu5AH cells do not appear to express a separate functional chylomicron remnant or apoE receptor. The apoB,E(LDL) receptors are responsible for β-VLDL and chylomicron remnant binding and uptake by these cells. Previously, Barnard, Erickson, and Cooper (38, 39) found that the rat hepatoma cell line HTC 7288c possesses apoB,E(LDL) receptors. These cells bound chylomicron remnants with high affinity (6 μg of chylomicron remnants/ml), although the question of whether the remnants were binding to remnant receptors, in addition to apoB,E(LDL) receptors, could not be resolved (38, 39).

Characterization of the $M_r \simeq 130{,}000$ apoB,E(LDL) receptors of the Fu5AH cells revealed both similarities to and differences from the apoB,E(LDL) receptors on human fibroblasts. In common with the human fibroblast, apoB,E(LDL) receptors on Fu5AH cells had the following properties: higher affinity for and approximately fourfold lower binding capacity for apoE- versus apoB-containing lipoproteins; Ca^{2+}-dependence for ligand binding; immunologic cross-reactivity with polyclonal antibodies against the bovine apoB,E(LDL) receptor and against the extreme carboxyl-terminal sequence of the human apo B,E(LDL) receptor; and a similar apparent molecular weight ($\simeq 130{,}000$) and orientation. However, three interesting and unusual properties of the apoB,E(LDL) receptor of the Fu5AH cells were discovered: an exceptionally low affinity for LDL; a propensity to undergo specific cleavage in Fu5AH cell homogenates; and a strong resistance to down-regulation after incubation of the cells with lipoproteins.

The affinity of Fu5AH cells for canine or rat LDL was approximately 10-fold lower than the corresponding affinity of LDL for rat fibroblast receptor (13). These data rule out the possibility that low apoB affinity was merely a reflection of poor cross-species receptor recognition of a nonhomologous LDL. The Fu5AH rat hepatoma cells are the first cell type that display an apoB,E(LDL) receptor with binding characteristics that are atypical of the fibroblasts of the same species.

A specific cleavage of the apoB,E(LDL) receptor of Fu5AH cells occurred in Fu5AH cell homogenates. The properties of the resulting receptor fragment—small reduction in size ($M_r \simeq 115{,}000$ vs. 130,000), retention of binding activity, release from cell membranes, and loss of reactivity with a carboxyl-terminal antibody—indicate an apparent cleavage site immediately external to the membrane spanning domain. Lehrman et al. (40) have recently described a mutation of the human apoB,E(LDL) receptor that involves a deletion of the carboxyl terminus and membrane spanning domain, and this deletion results in the secretion of a truncated receptor protein that is analogous to the receptor fragment generated in Fu5AH cells. Presumably Fu5AH cells contain a very active protease with a specific cleavage site on the apoB,E(LDL) receptor, and presumably this protease is released upon cell disruption. However, the absence of the receptor fragment from Triton extracts of Fu5AH cells suggests that there is little or no accumulation of this fragment in intact cells. Previously, Cooper, Nutik, and Chen (41) found trace amounts of an $M_r \simeq 115{,}000$ protein from rat liver membranes that reacted with rat apoB,E(LDL) receptor antibody. They suggested this protein was an apoB,E(LDL) receptor precursor, but, most likely, a small amount of proteolytic product of the mature protein was formed during the isolation of the membranes similar to the $M_r \simeq 115{,}000$ protein observed in this study.

The resistance to down-regulation is probably the most important feature of the apoB,E(LDL) receptor on Fu5AH cells because of its potentially direct relevance to cholesteryl ester accumulation. A delay in or suppression of receptor down-regulation, by allowing continued uptake of lipoprotein-derived cholesterol and cholesteryl esters, could lead directly to increased deposition of cholesteryl esters. Other cell types that undergo foam cell formation and have a relative resistance to receptor down-regulation are the J774 mouse macrophage-like cell line (42) and mouse peritoneal macrophages (12).

Several possible mechanisms for the decreased sensitivity to down-regulation can be envisioned. The massive accumulation of cholesteryl ester implies very high acyl CoA:cholesterol acyltransferase (ACAT) activity in Fu5AH cells. If unesterified cholesterol is necessary for apoB,E(LDL) receptor regulation, then a high level of ACAT activity could limit receptor down-regulation by diversion of free cholesterol to the cholesteryl ester pool in a manner similar to that postulated for J774 mouse macrophages (43). Alternatively, a difference in the apoB,E(LDL) receptor itself or a difference in the receptor gene promoter or regulatory proteins could account for the resistance to down-regulation. It is likely that mechanisms unique to the liver allow differential regulation of hepatic lipoprotein receptors. The Fu5AH cells may provide clues as to how these mechanisms function.

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