Abnormal high density lipoproteins from patients with liver disease regulate cholesterol metabolism in cultured human skin fibroblasts[1]

James S. Owen,[2] Harry Goodall, Pramod Mistry, David S. Harry, Richard C. Day, and Neil McIntyre

Academic Department of Medicine, Royal Free Hospital School of Medicine (University of London), Hampstead, London NW3 2PF, United Kingdom

Abstract Apolipoprotein B (apoB) of plasma low density lipoproteins (LDL) binds to high affinity receptors on many cell types. A minor subclass of high density lipoproteins (HDL), termed HDL_1, which contains apoE but lacks apoB, binds to the same receptor. Bound lipoproteins are engulfed, degraded, and regulate intracellular cholesterol metabolism and receptor activity. The HDL of many patients with liver disease is rich in apoE. We tested the hypothesis that such patient HDL would reduce LDL binding and would themselves regulate cellular cholesterol metabolism. Normal HDL had little effect on binding, uptake, and degradation of ^{125}I-labeled LDL by cultured human skin fibroblasts. Patient HDL (d 1.063–1.21 g/ml) inhibited these processes, and in 15 of the 25 samples studied there was more than 50% inhibition at ^{125}I-labeled LDL and HDL protein concentrations of 10 μg/ml and 25 μg/ml, respectively. There was a significant negative correlation between the percentage of ^{125}I-labeled LDL bound and the apoE content of the competing HDL ($r = -0.54$, $P < 0.01$). Patient ^{125}I-labeled HDL was also taken up and degraded by the fibroblasts, apparently through the LDL-receptor pathway, stimulated cellular cholesterol esterification, increased cell cholesteryl ester content, and suppressed cholesterol synthesis and receptor activity. We conclude that LDL catabolism by the receptor-mediated pathway may be impaired in liver disease and that patient HDL may deliver cholesterol to cells.—**Owen, J. S., H. Goodall, P. Mistry, D. S. Harry, R. C. Day, and N. McIntyre.** Abnormal high density lipoproteins from patients with liver disease regulate cholesterol metabolism in cultured human skin fibroblasts. *J. Lipid Res.* 1984. **25:** 919–931

Supplementary key words apoE • LDL-receptor

Cholesterol is an essential structural component of many cellular membranes and can be synthesized by most cells. But many cells also obtain cholesterol by uptake of low density lipoproteins (LDL), the major carrier of plasma cholesterol (2). Goldstein and Brown (3) showed that cultured cells have high affinity receptors, located in clathrin-coated pits on the surface membrane, which recognize the apolipoprotein moiety (apoB) of LDL. Following binding, LDL is engulfed as intact particles and transported to lysosomes where the cholesteryl ester is hydrolyzed and apoB is degraded. The LDL-derived cholesterol affects cellular cholesterol content in several ways (2). There is enhanced activity of cellular acyl-CoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26) which esterifies excess cholesterol for storage as cholesteryl ester, cell cholesterol synthesis is inhibited, and production of receptors is switched off so that further entry of LDL into cells is reduced.

The receptors for LDL are not specific for apoB. Innerarity and Mahley (4) and Pitas et al. (5) showed that cultured cells bind apoE-rich lipoproteins with a greater affinity than LDL containing apoB. Following binding, apoE-rich particles are taken up by the cells and regulate intracellular cholesterol metabolism and receptor activity in much the same way as LDL (6, 7). The HDL fraction in patients with liver disease is often rich in apoE (8, 9). In this study we have tested the hypothesis that addition of such abnormal patient HDL to cultured cells would inhibit LDL catabolism and that patient HDL would itself regulate cellular cholesterol metabolism.

MATERIALS AND METHODS

Patients

HDL was isolated from twenty-three in-patients with liver disease of varying etiology and severity (**Table 1**).

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase (EC 2.3.1.26); apo, apolipoprotein; DMEM, Dulbecco's modified Eagle's medium; HDL, high density lipoproteins; HEPES, 4-(2-hydroxyethel)-1-piperazine-ethanesulfonic acid; LCAT, lecithin:cholesterol acyltransferase (EC 2.3.1.43); LDL, low density lipoproteins; LPDS, lipoprotein-deficient serum; PBS, Dulbecco's phosphate-buffered saline; PBS/BSA, PBS containing 2 mg/ml bovine serum albumin; SDS, sodium dodecyl sulfate.

[1] Part of this study was presented at the International Association for the Study of the Liver Meeting in Chicago (1).

[2] To whom correspondence should be addressed.

TABLE 1. Clinical data of patients with liver disease

Patient	Age	Sex	Diagnosis	Bilirubin	Aspartate Transaminase	Alkaline Phosphatase	Albumin	HDL Apolipoprotein Composition[a]			^{125}I-LDL Bound[b]
								pI 6.3	pI 6.0	apoE	
	yr			*μmol/l*	*I.U./l*	*I.U./l*	*g/l*		%		%
1	54	F	Primary biliary cirrhosis	210	305	1180	35	3.0	3.0	18.0	4
2	50	F	Primary biliary cirrhosis	92	47	97	40	9.6	9.6	14.0	10
3	56	F	Primary biliary cirrhosis	555	110	220	44	0	0	9.1	13
4	59	F	Chronic active hepatitis	66	52	24	31	1.3	0	20.2	14
1	53	F	Primary biliary cirrhosis	228	136	150	48	3.7	3.0	6.7	16
5	49	F	High biliary stricture	72	81	88	34	2.6	0	10.5	17
3	56	F	Primary biliary cirrhosis	480	130	180	43	8.7	2.8	9.4	24
6	55	F	Alcoholic hepatitis	40	21	17	28	6.2	0	10.5	24
7	51	F	Alcoholic cirrhosis	47	36	19	32	7.0	6.9	9.9	26
8	53	F	Primary biliary cirrhosis	230	75	93	48	7.7	5.0	15.1	28
9	25	M	Chronic active hepatitis	77	33	42	33	8.9	7.8	14.2	33
10	77	F	Carcinoid syndrome	10	55	23	23	4.6	0	15.3	41
11	54	M	Alcoholic cirrhosis	45	34	34	38	0	0	20.2	46
12	52	M	Chronic active hepatitis	46	24	8	37	4.0	5.1	15.0	46
13	46	M	Chronic active hepatitis	18	40	11	27	2.5	4.0	5.1	47
14	39	M	Cryptogenic cirrhosis	49	49	72	40	0	0	3.0	53
15	67	M	Cholestasis 2° to infection	41	47	40	31	8.8	5.3	10.1	56
16	37	M	Chronic active hepatitis	9	30	19	29	4.2	0	13.7	57
17	47	F	Alcoholic hepatitis	33	26	17	40	0	2.0	3.0	61
18	58	F	Chronic active hepatitis	63	47	20	31	0	0	11.9	80
19	54	M	Alcoholic cirrhosis	36	35	10	40	0	0	3.0	83
20	51	M	Alcoholic cirrhosis	34	33	67	41	1.6	3.0	8.0	84
21	73	F	Alcoholic hepatitis	21	25	17	34	0	0	3.0	85
22	40	M	Alcoholic cirrhosis	17	36	17	39	0	0	7.1	86
23	23	F	Chronic active hepatitis	51	71	12	43	0	0	4.0	89

[a] Apolipoproteins from delipidated patient HDL were separated by isoelectric focusing and relative amounts were measured by densitometry; pI 6.3 and pI 6.0 refer to the two unidentified apolipoproteins with these pI values (see Results section).

[b] The percentage of ^{125}I-labeled LDL bound by cultured skin fibroblasts relative to controls when incubated at a protein concentration of 10 μg/ml in the presence of 25 μg protein/ml of HDL from patients with liver disease.

Diagnosis was established in various ways including liver biopsy, cholangiography, and surgery. Seven patients had obstructive jaundice (six, intrahepatic; one, extrahepatic) and sixteen had parenchymal liver disease (seven, chronic active hepatitis; five, alcoholic cirrhosis; three, alcoholic hepatitis; one, cryptogenic cirrhosis). Two of the patients with intrahepatic obstructive jaundice (both primary biliary cirrhotics) were studied on two occasions, separated by 9 months in one case (patient 3) and by 17 months in the other (patient 1). Comparison HDL was obtained from ten healthy medical and laboratory staff members. Informed verbal consent was obtained from all patients prior to blood withdrawal.

Reagents

Fetal calf serum was obtained from Gibco Europe, Paisley, Scotland; all other tissue culture materials were from Flow Laboratories Ltd., Irvine, Scotland. Sodium heparin, grade II, was supplied by Sigma Chemical Co. Radioactive chemicals were obtained from Amersham International Ltd., Amersham, Bucks, U.K. All solvents were redistilled before use.

Lipoproteins and lipoprotein-deficient serum

LDL (d 1.019–1.063 g/ml) from normal subjects and HDL (d 1.063–1.21 g/ml) from both patients and normal subjects were isolated by sequential preparative ultracentrifugation of fresh plasma (10). Density adjustments were made with NaCl and NaBr and ultracentrifugation was carried out at 16°C in an 8 × 50 ml angle-head rotor using either an MSE Superspeed 65 or an MSE Prepspin 50 centrifuge (MSE Scientific Instruments, Sussex, U.K.). Lipoprotein-deficient serum (LPDS) was prepared by ultracentrifugation of serum from normal subjects at a density of 1.215 g/ml. All fractions were washed by recentrifugation at the appropriate density and then dialyzed against 10 mM sodium phosphate buffer, pH 7.4, containing 145 mM NaCl and 0.3 mM disodium EDTA. The protein concentration of the LPDS was adjusted to 50 mg/ml; it contained less than 2 μg/ml of cholesterol. Some isolated HDL fractions were characterized further by chemical analysis and by electron microscopy as described previously (11, 12).

Apolipoprotein composition was analyzed following

delipidation of the lipoproteins with ethanol–ether 3:1 (v/v). Individual apolipoproteins were separated by isoelectric focusing (pH 4.0–6.5) (13); the gels were stained with Coomassie blue and scanned in a Quick Scan Jr. densitometer (Helena Laboratories, TX). Relative amounts of individual apolipoproteins present were calculated from the peak areas and expressed as a percentage of the total. In certain cases separation of apolipoproteins was also carried out by SDS and/or urea polyacrylamide gel electrophoresis (14).

Partial delipidation with diethyl ether (4) and cyclohexanedione modification of apolipoprotein arginine residues of patient HDL were accomplished as described by Mahley and colleagues (15). Normal LDL and normal HDL were labeled with ^{125}I immediately after isolation using the method of MacFarlane (16). Patient HDL was iodinated by the procedure of Bolton and Hunter (17) as described for lipoproteins by Innerarity, Pitas, and Mahley (18). The newly labeled lipoproteins were separated from the reaction products by chromatography on Sephadex G-10, followed by extensive dialysis to remove residual non-bound iodide. Immediately before addition to the cultured cells, ^{125}I-labeled and unlabeled lipoproteins were passed through a 0.2-μm filter; their protein concentration was measured by the method of Lowry et al. (19) using bovine serum albumin as standard. Lipoprotein concentrations are expressed as μg of protein per ml unless otherwise stated and all values refer to final concentrations in the incubation medium.

Cell culture

The fibroblasts used were established from skin biopsies of normal subjects and maintained in monolayer cultures by standard procedures (20, 21). The cells were grown in a humidified incubator (5% CO_2 in air) at 37°C in 75-cm^2 flasks and routinely split 1 to 6, once a week, by dissociating the confluent monolayer of cells with 0.05% trypsin and 0.02% EDTA. Growth medium was Dulbecco's modified Eagle's media (DMEM), supplemented with 10% (v/v) fetal calf serum, 100 units/ml of potassium penicillin G, and 100 μg/ml of streptomycin sulfate, and cells were used between the 5th and 15th passage.

All experiments were carried out using a standard protocol (20, 21). Cells from the stock cultures were transferred to either 60-mm diameter Petri dishes (10^5 cells/dish) or 100-mm diameter Petri dishes (2×10^5 cells/dish) and grown in the media described above. On the 6th day, when the cells were almost confluent, the media was removed and the cells were washed twice with Dulbecco's phosphate-buffered saline (PBS) before addition of either 2 ml (60-mm dishes) or 5 ml (100-mm dishes) of DMEM containing 10% (v/v) LPDS and antibiotics. Experiments with the lipoprotein fractions were begun 24 hr later.

Assays for binding, internalization, and degradation

The ability of unlabeled normal and patient HDL (at final protein concentrations of 5, 10, 25, and 50 μg/ml) to compete with 10 μg/ml of ^{125}I-labeled LDL for cellular binding, internalization, and degradation was determined during 4-hr incubations in 60-mm Petri dishes at 37°C. After the incubation period, the dishes were placed on ice and the medium was removed for the degradation assay (20). The cells were washed seven times with ice-cold PBS containing 2 mg/ml bovine serum albumin (PBS/BSA) and three times with PBS. The ^{125}I-labeled LDL bound to high-affinity sites was released by addition of ice-cold heparin solution (50 mM NaCl/10 mM HEPES/1% (w/v) heparin) followed by gentle shaking of the dishes for 60 min at 4°C (22). The heparin solution was then removed for assay of ^{125}I (Wallac 1280 Ultragamma counter) and the cells were washed twice with PBS/BSA and three times with PBS. The cell monolayer was dissolved by shaking with 0.1 M NaOH for at least 30 min at room temperature and portions were removed for the determination of total cell protein and ^{125}I radioactivity. The latter represents internalized ^{125}I-labeled LDL. The radioactive acid-soluble non-iodide degradation products of LDL catabolism were measured in the incubation media after precipitation with trichloroacetic acid (10% (w/v) final concentration), oxidation by H_2O_2, and two extractions with chloroform (20).

When binding of ^{125}I-labeled patient HDL was studied less than 20% of the surface-associated radioactivity was released by heparin, presumably because of its high apoE content (4). Therefore, bound ^{125}I-labeled patient HDL or normal HDL were released from the cells by incubation with 0.05% trypsin-0.02% EDTA for 5–6 min at 37°C (23). The reaction was stopped by addition of DMEM containing 10% fetal calf serum and the dishes were placed on ice. The cell suspension was centrifuged (1000 *g*) for 10 min at 4°C and a portion of the supernatant was removed for assay of ^{125}I. The cell pellet was washed twice with PBS, dissolved in 1 M NaOH, and portions were taken for protein and ^{125}I estimations.

All binding, internalization, and degradation assays were carried out in duplicate. The nonspecific contribution to these processes was determined in parallel duplicate dishes containing 1 mg protein/ml of unlabeled LDL; these values were then subtracted from each of the experimental results to give specific receptor-mediated binding, internalization, and degradation of the labeled lipoproteins.

[^{14}C]Sucrose uptake (fluid endocytosis)

To determine whether any increase in cellular uptake of patient HDL could be attributed to enhanced non-adsorptive pinocytosis (fluid endocytosis), we measured uptake of radioactive sucrose by the fibroblasts (24). After 24 hr incubation with the LPDS-medium, 1 μCi of [U-^{14}C]sucrose (sp act 434 mCi/mmol) was added together with test lipoproteins and the incubation was continued for a further 4 hr. The cells were washed twice with PBS/BSA and three times with PBS, dissolved in 0.1 M NaOH, and portions were taken for ^{14}C measurement and protein estimation. Clearances of [^{14}C]sucrose (μl medium/mg cell protein) were calculated by dividing the uptake of radioactivity into the cells (dpm/mg cell protein) by the concentration of radioactivity in the medium (dpm/μl).

Cellular cholesterol and cholesteryl ester concentrations

Cells in 100-mm Petri dishes were washed twice with PBS, following a 24-hr incubation with LPDS-medium. Test lipoproteins were added in fresh LPDS-medium and the incubation was continued for a further 24 hr. The cells were washed twice with PBS/BSA and three times with PBS. They were then scraped into glass tubes with a plastic spatula, pelleted by centrifugation (1000 *g* for 10 min), and lysed with 0.4 ml of water. Cell lipids were extracted with 3 ml of chloroform–methanol 1:2 (v/v) containing 10^5 dpm of [^{14}C]oleic acid as internal standard. The denatured protein was pelleted by centrifugation and the supernatant was transferred to clean tubes. The protein pellet was dissolved in 1 M NaOH and a portion was taken for protein estimation. One ml of chloroform and 1 ml of water were added to the supernatant, the contents were mixed, and the lower phase was applied to silica gel G thin-layer chromatography plates. The lipid classes were separated by developing the plates in hexane–diethyl ether–acetic acid 90:20:1 (by vol). The fractions were located by brief exposure to iodine vapor; the fraction corresponding to unesterified fatty acids was scraped from the plate and its ^{14}C radioactivity was determined to allow calculation of fractional recoveries. The cholesterol and cholesteryl ester fractions were removed and hydrolyzed at 80°C for 1 hr with 2 ml of 1 M ethanolic KOH as described previously (25). Water (2.5 ml) was added and cholesterol was extracted into 2.5 ml of hexane containing 5 μg/ml 5α-cholestane as an internal standard. The hexane layer was evaporated to dryness under nitrogen, the sterols were dissolved in 50 μl of cyclohexane and 4-μl portions were used to determine the cholesterol content by gas–liquid chromatography with a 5840A Hewlett-Packard Gas Chromatograph. Separation was at 265°C in a 3-foot column of 3% OV-17 on Chromosorb W; detection was by flame ionization and the cholesterol concentration was calculated using the internal standard/area ratio technique.

Measurement of cellular sterol synthesis, cholesterol esterification, and LDL receptor activity

These experiments were carried out with cells grown in 60-mm dishes using the same standard format. After 24-hr incubation with LPDS-medium as described above, the medium was removed, replaced with fresh LPDS-medium containing the test lipoproteins, and incubated a further 24 hr.

To estimate cellular sterol synthesis, 2 μCi of [2-^{14}C]acetate (sp act 57 mCi/mmol) was added to each dish and incubation was continued for 1 hr. The cells were washed twice with PBS/BSA and three times with PBS. They were dissolved in 2 ml of 0.1 M NaOH and 0.5 ml was removed for protein estimation. One ml was added to a Teflon-lined, screw-capped tube containing 1 ml of ethanol and 0.2 ml of 90% KOH. The mixture was saponified by heating for 3 hr at 80°C, diluted with 1.5 ml of water, and the nonsaponifiable lipids were extracted into 2.5 ml of hexane (25). The hexane layer was washed once with 2.5 ml of 0.1 M sodium acetate and a portion was taken for radioactivity measurement. The results given for sterol synthesis, therefore, refer to the incorporation of ^{14}C radioactivity into nonsaponifiable lipids.

Cellular cholesterol esterification was measured by incorporation of [9,10-^{3}H]oleic acid into cholesteryl esters. The radioactive oleic acid was dissolved in acetone and bound to defatted human serum albumin in a 10% (w/v) solution as described by Stokke and Norum (26) for complexing radioactive cholesterol. Five μCi of the [9,10-^{3}H]oleic acid (diluted with unlabeled oleic acid to a specific activity of 25 mCi/mmol) was added to each dish and the incubation was continued for 2 hr. The cells were then washed as before, scraped into glass tubes with a plastic spatula, and pelleted by centrifugation. Water (0.4 ml) was added, followed by 3 ml of chloroform–methanol 1:2 (v/v) containing 10^5 dpm of [^{14}C]cholesterol as internal standard, to extract the lipids. Protein estimation of the denatured protein pellet and separation of the [^{3}H]cholesteryl esters and [^{14}C]cholesterol by thin-layer chromatography was as described above for mass measurements. Cellular ACAT activity was expressed as picomoles of cholesteryl [^{3}H]oleate formed per mg of cell protein.

Cultured fibroblasts chilled to 0–4°C are capable of binding LDL to receptor sites, but are unable to engulf the bound LDL particles (22). This method was used to evaluate cellular LDL receptor activity after 24-hr in-

cubations with test lipoproteins. The cells were washed five times as described above, except that the wash solutions were at 37°C in order to internalize lipoproteins bound to the surface (7), and then placed on ice for 15 min. The PBS medium was removed and replaced by ice-cold DMEM containing 20 mM HEPES (pH 7.4), instead of sodium bicarbonate, 10% LPDS, and 7.5 μg of protein/ml of ^{125}I-labeled LDL. Incubations were carried out for 2 hr at 4°C with gentle shaking. The medium was removed and the cells were washed seven times with PBS/BSA and three times with PBS. The cell monolayer was then dissolved in 0.1 M NaOH and portions were taken for measurement of ^{125}I radioactivity and protein.

RESULTS

Competition studies

The ability of unlabeled LDL, normal HDL, and HDL from a patient with chronic active hepatitis to affect the binding, internalization, and degradation of ^{125}I-labeled LDL by cultured, human skin fibroblasts is shown in **Fig. 1.** The competition curves shown for normal HDL and LDL are representative of our experiments and are in agreement with those reported by other workers (12, 27). Unlabeled LDL readily reduced the binding, internalization, and degradation of 10 μg of protein/ml of ^{125}I-labeled LDL with 50% inhibition at about 20–25 μg protein/ml. Unlabeled normal HDL had only a small inhibitory effect on these processes. The inhibition by patient HDL was variable; some patient HDL was much more inhibitory than unlabeled LDL as shown by the competition curves in Fig. 1; other HDL samples were similar to normal HDL and had only moderate ability to compete. At a concentration of 25 μg protein/ml, patient HDL was able to reduce binding of the ^{125}I-labeled LDL to a mean of 45 ± 27% (mean ± SD, range 10–89%) compared to 86 ± 9% by the normal HDL ($P < 0.001$) (**Fig. 2**). The mean percentages of ^{125}I-labeled LDL internalized and degraded in the presence of unlabeled patient HDL were similar to that of ^{125}I-labeled LDL bound (Fig. 2).

Patient HDL was also able to inhibit binding of LDL at 4°C, when little internalization or degradation can occur (**Fig. 3A**). Modification of the arginyl residues of the apolipoproteins of patient HDL with cyclohexanedione reduced their ability to prevent uptake of ^{125}I-labeled LDL (Fig. 3B). However, partial delipidation of patient HDL which removed most of the neutral lipids had a negligible effect on their inhibitory ability (Fig. 3C).

Apolipoprotein analysis

The apolipoproteins of patient HDL were separated by isoelectric focusing and their distribution was com-

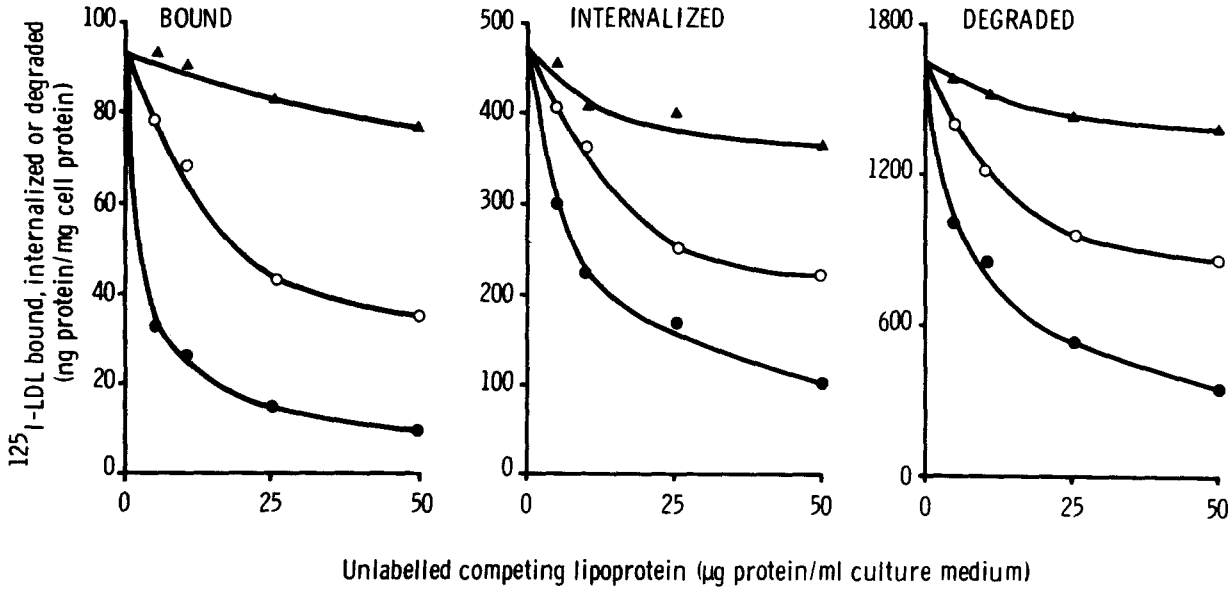

Fig. 1. Ability of unlabeled lipoproteins to inhibit binding, internalization, and degradation of ^{125}I-labeled LDL by cultured skin fibroblasts. Cell monolayers were preincubated for 24 hr in medium containing 10% LPDS. ^{125}I-Labeled LDL (10 μg of protein/ml) was then incubated with increasing protein concentrations (5, 10, 25, and 50 μg/ml) of normal LDL (○), normal HDL (▲), or HDL from a patient (# 4) with chronic active hepatitis (●) for 4 hr at 37°C. Following the incubation period, the fibroblasts were analyzed for bound and internalized ^{125}I-labeled LDL, and the culture medium for degradation products as described in the Materials and Methods section. Each point is the mean of duplicate dishes and was corrected for nonspecific contributions by addition of excess unlabeled LDL (1 mg of protein/ml) in parallel experiments.

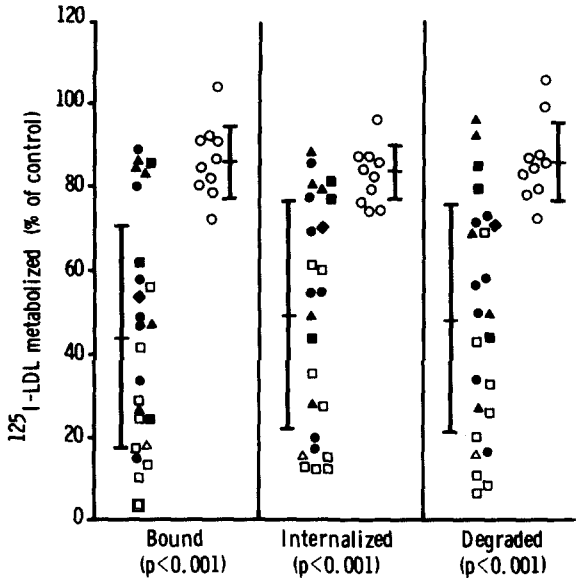

Fig. 2. Ability of liver disease HDL to inhibit ^{125}I-labeled LDL catabolism by cultured skin fibroblasts. Incubation conditions were as described in Fig. 1 and the ^{125}I-labeled LDL bound, internalized, and degraded was extrapolated from the competition curves when either liver disease HDL or normal HDL (○) were present at 25 μg protein/ml. Liver disease HDL was isolated from patients with extrahepatic jaundice (△), intrahepatic obstructive jaundice (□), chronic active hepatitis (●), alcoholic cirrhosis (▲), alcoholic hepatitis (■), and cryptogenic cirrhosis (♦). The results were obtained from seven independent experiments and are expressed as a percentage of control dishes; the bars indicate mean ± SD.

pared to those in normal HDL (Table 1 and **Fig. 4A**). In agreement with other studies (8, 9) the mean apoE content of patient HDL (10.4 ± 5.3, range 3.0–20.2%) was higher than that in normal HDL (3.9 ± 1.0, range 2.6–5.4%) ($P < 0.001$). Surprisingly, seventeen of the twenty-five patient HDL fractions studied contained one or two additional, more cathodic apolipoproteins not detected in normal HDL (Table 1). They were glycoproteins with pI values of 6.3 and 6.0 (Fig. 4A), molecular weights of about 22,000 and 18,000 as estimated on SDS-gels (Fig. 4B), and electrophoretic mobilities between apoA-I and apoA-II on urea gels (Fig. 4C); they did not react with antisera to apoE, apoA-I, or normal HDL. Of the patients studied twice, # 1 contained the additional apolipoproteins on both occasions and # 3 only on the first occasion (Table 1). Further properties and characteristics of these apolipoproteins will be published elsewhere.

Of the seventeen patient HDL fractions with the additional apolipoproteins only three failed to inhibit ^{125}I-labeled LDL uptake and degradation by 50% or more at 25 μg protein/ml; of the eight without these apolipoproteins, seven inhibited these processes by less than 50%. There were significant inverse correlations between the percentage of ^{125}I-labeled LDL bound and the proportion of both apoE ($r = -0.54$, $P < 0.01$) and additional apolipoproteins ($r = -0.47$, $P < 0.05$) in the competing patient HDL when added at 25 μg protein/ml (as calculated from the results shown in Table 1).

Studies with ^{125}I-labeled patient HDL

Three patient HDL fractions were labeled with ^{125}I as described in the Methods section. Specific activities were 617–1144 cpm/ng protein; less than 6% of the total ^{125}I radioactivity was TCA-soluble and less than 9% was extractable into isopropanol–chloroform 11:7

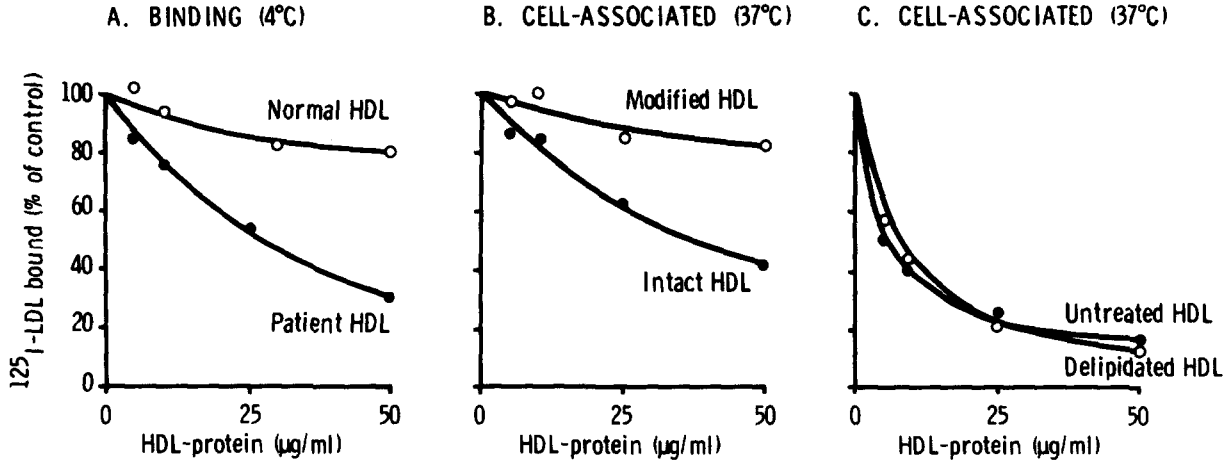

Fig. 3. Competitive inhibition of ^{125}I-labeled LDL binding, or binding plus internalization, to cultured skin fibroblasts by modified or untreated liver disease HDL. Panel A, incubations were for 24 hr at 4°C with 10 μg protein/ml of ^{125}I-labeled LDL (164 cpm/ng protein) and increasing concentrations of either normal HDL (○) or HDL from a patient (# 14) with cryptogenic cirrhosis (●). Control value was 96 ng of ^{125}I-labeled LDL bound per mg of cell protein. Panel B, incubations were for 4 hr at 37°C with 10 μg protein/ml of ^{125}I-labeled LDL (164 cpm/ng protein) and increasing concentrations of HDL from a patient (# 16) with chronic active hepatitis (○) or the same patient HDL modified by treatment with cyclohexanedione (●). Control value was 580 ng of ^{125}I-labeled LDL protein bound and internalized per mg of cell protein. Panel C, incubation conditions were as in panel B, except that the competing HDL was from a patient (# 3) with primary biliary cirrhosis and was either untreated (○) or partially delipidated (●) before addition to the culture medium. Partial delipidation of the patient HDL removed 89% of the cholesterol, 22% of phospholipids, and almost all of the triglyceride and cholesteryl ester.

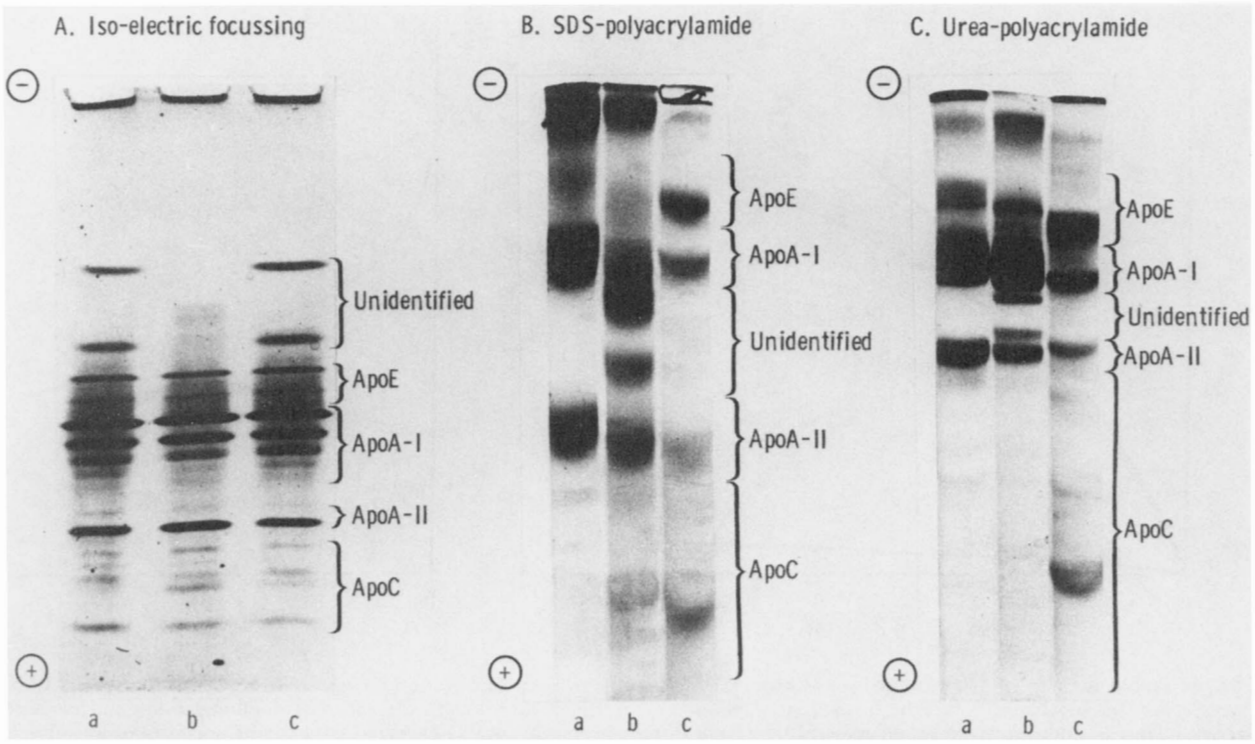

Fig. 4. Isoelectric focusing and polyacrylamide gel electrophoresis of delipidated HDL from patients with liver disease. Panel A, samples of delipidated patient HDL (approximately 50 μg of total protein) were applied to polyacrylamide gels containing 3% ampholines (pH 4.0–6.5) and subjected to electrophoresis for 16 hr at 250 V. Lane a, HDL from a patient (# 3) with primary biliary cirrhosis; it reduced ^{125}I-labeled LDL binding to 24% of control values and the apolipoproteins contained 9.4% apoE and 11.5% of the two unidentified apolipoproteins (Table 1). Lane b, HDL from a patient (# 22) with alcoholic cirrhosis; it reduced LDL binding to 86% and the apolipoproteins contained 7.1% apoE. Lane c, HDL from a patient (# 7) with alcoholic cirrhosis; it reduced LDL binding to 26% and the apolipoproteins contained 9.9% apoE and 13.9% unidentified apolipoproteins. The apolipoprotein pattern of normal HDL was invariably the same as that shown in lane b, except that the apoE content was always less than 6%. Panel B, electrophoretic patterns of delipidated HDL (approximately 50 μg of total protein) in 7.5% polyacrylamide gels containing 0.1% SDS and 8 M urea at pH 7.0. Examples of HDL apolipoproteins from a normal subject and patients # 3 and # 11 are shown in lanes a, b, and c, respectively. Panel C, the apolipoprotein samples and the electrophoresis conditions were as described for panel B, except that the SDS was omitted and the pH was 8.4.

(v/v) (28). After separation by isoelectric focusing, the mean distribution of radioactivity in the apolipoproteins was 43% in apoA-I, 34% in apoA-II, 16% in C apolipoproteins, 5.5% in apoE, and 1.5% in the additional apolipoproteins. The integrity of ^{125}I-labeled patient HDL was assessed in one preparation by diluting it up to 15-fold with unlabeled patient HDL, while maintaining a constant protein concentration of 20 μg/ml, and testing whether the reductions in binding, internalization, and degradation of the patient ^{125}I-labeled HDL by the cells were theoretical. All three processes were decreased in direct proportion to the extent of isotope dilution, suggesting that the cultured skin fibroblasts did not distinguish between labeled and unlabeled patient HDL. Similar results have been found for normal LDL (29) and normal HDL (27).

Binding curves at 4°C for ^{125}I-labeled patient and normal HDL (trypsin-releasable radioactivity), and for ^{125}I-labeled normal LDL (heparin-releasable radioactivity) are shown in **Fig. 5A.** In agreement with the results of Goldstein et al. (22), binding of LDL by fibroblasts increased with LDL concentration in a nonlinear manner. Such binding of LDL has been adequately described in terms of two binding sites: one of high affinity and saturable at about 5–10 μg of LDL protein/ml (the 'LDL-receptor'), the other of low affinity and apparently nonsaturable (20, 22). The fibroblasts bound patient HDL in a similar manner to LDL with evidence for both high and low affinity binding sites. Much greater amounts of patient HDL were bound with high affinity than normal HDL. Unlabeled LDL readily displaced ^{125}I-labeled patient HDL from the binding sites on the cells (Fig. 5B). The competitive effects of unlabeled HDL are not shown because these would have been partly indirect; the specific activity of ^{125}I-labeled patient HDL would have decreased during the incubation period as a consequence of apolipoprotein exchange between unlabeled patient HDL or unlabeled normal HDL and labeled patient HDL.

The time course for cell association (i.e., binding plus internalization) and degradation of normal ^{125}I-labeled LDL, normal ^{125}I-labeled HDL, and patient ^{125}I-labeled

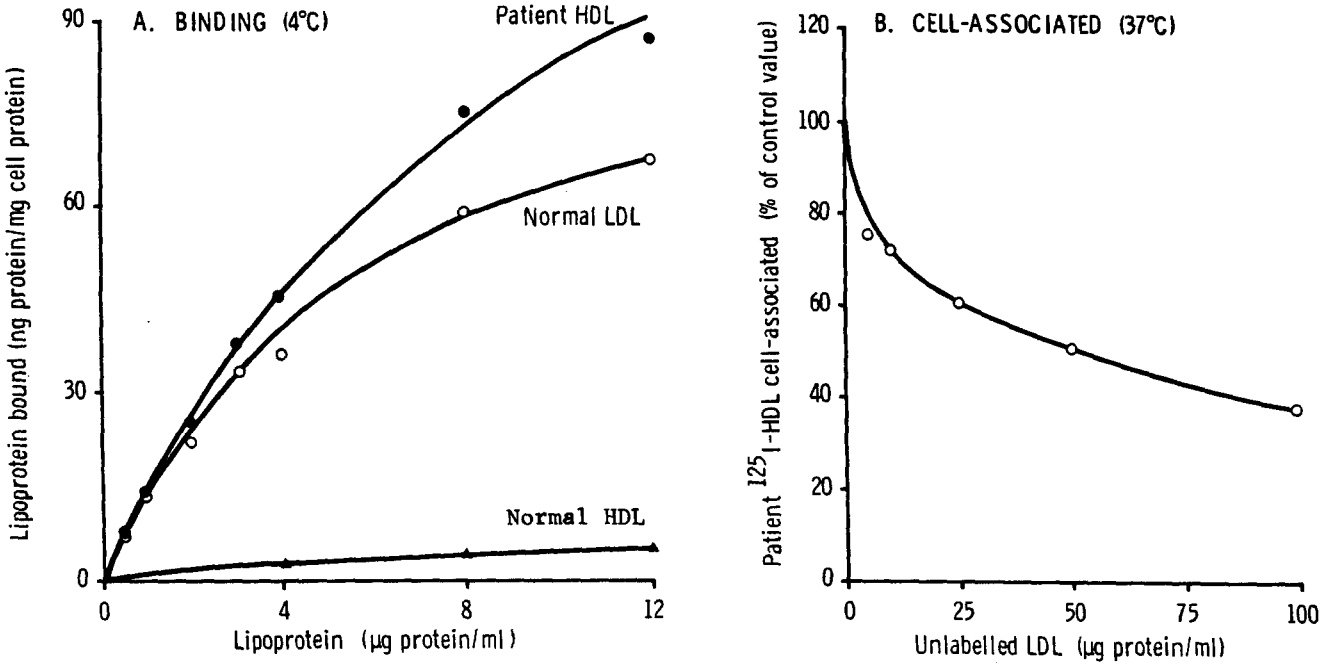

Fig. 5. Saturable binding of ^{125}I-labeled liver disease HDL by cultured skin fibroblasts and its displacement by unlabeled LDL. Cell monolayers were preincubated for 24 hr in medium containing 10% LPDS. Results are the mean of duplicate dishes and corrected for nonspecific effects by addition of excess unlabeled LDL (1 mg of protein/ml) in parallel experiments. Panel A, incubations were for 2 hr at 4°C with ^{125}I-labeled HDL from patient # 2 with primary biliary cirrhosis (●, 617 cpm/ng protein), normal HDL (▲, 712 cpm/ng protein), or normal LDL (O, 312 cpm/ng protein). The patient HDL, unlabeled, reduced ^{125}I-labeled LDL binding to 10% of control values (Table 1); it contained 19.2% of unidentified apolipoproteins and 14.0% apoE following delipidation. Bound patient HDL and normal HDL were released by trypsinization of the cells, and normal LDL by treatment with heparin as described in the Materials and Methods section. Panel B, incubations were for 4 hr at 37°C with 5 μg of protein/ml of ^{125}I-labeled HDL from a patient (# 4) with chronic active hepatitis (824 cpm/ng protein) and normal LDL (O). Control value was 390 ng of ^{125}I-labeled patient HDL bound and internalized per mg of cell protein. Unlabeled HDL from patient # 4 reduced ^{125}I-labeled LDL binding to 14% of control values (Table 1); it contained 20.2% apoE and 1.3% unidentified apolipoproteins following delipidation.

HDL during incubation with skin fibroblasts at 37°C is shown in **Figs. 6A** and **6B,** respectively. The amount of each lipoprotein bound and internalized by the cells began to reach a plateau after 2–3 hr and showed little increase on further incubation. By contrast, after an initial short lag period, degradation of the lipoproteins increased steadily throughout the experimental period (12 hr). Much less normal HDL was taken up and

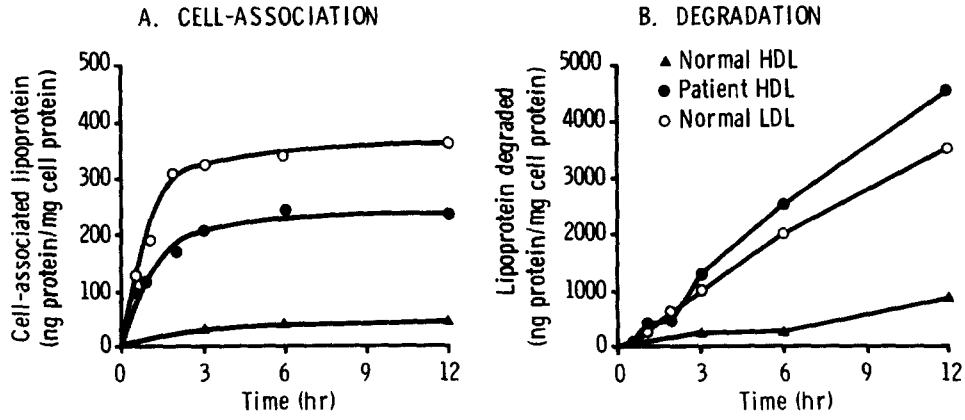

Fig. 6. Time course of binding plus uptake and of degradation of liver disease HDL by cultured skin fibroblasts. Cell monolayers were preincubated for 24 hr in medium containing 10% LPDS and then at 37°C for 0.5–12 hr with 5 μg of protein/ml of the labeled lipoproteins described in Fig. 5A. Patient HDL (●), normal HDL (▲), and normal LDL (O). Each point is the mean of duplicate dishes and was corrected for nonspecific effects by addition of excess unlabeled LDL (1 mg protein/ml) in parallel experiments.

degraded by the cells than either normal LDL or patient HDL.

Cellular uptake of [^{14}C]sucrose

The enhanced uptake of patient HDL by fibroblasts compared to normal HDL may have reflected stimulation of nonspecific endocytosis rather than a receptor-mediated process. To test this possibility [^{14}C]sucrose was added to the incubation medium and its uptake was measured in the absence and presence of lipoproteins. Uptake of sucrose by the cells over over 4 hr was low, corresponding to 3.0 μl of medium cleared (**Table 2**). This clearance rate was not altered when patient HDL, normal HDL, or normal LDL were added to the incubation medium at protein concentrations of either 5 μg/ml or 50 μg/ml. The clearance of patient ^{125}I-labeled HDL at 5 μg/ml, as estimated by its uptake and degradation (Fig. 6) was more than 100-fold greater than that of sucrose.

Patient HDL and cellular cholesterol metabolism

The uptake and degradation of patient HDL by the fibroblasts resembled that of normal LDL. Several experiments were carried out to establish whether patient HDL, like normal LDL, could also affect total cell cholesterol content and regulate intracellular cholesterol metabolism. Cell cholesterol and cholesteryl ester contents were measured 24 hr after incubation with various lipoproteins at equal total cholesterol concentrations. Addition of normal LDL to the incubation medium raised the mean cholesteryl ester content from 2 μg/mg cell protein to 16 μg, whilst patient HDL produced a rise to 6 μg (**Table 3**). By contrast, addition of normal HDL did not change the cholesteryl ester content of the cells.

Both patient HDL and normal LDL stimulated cellular cholesterol esterification (**Fig. 7A**) and suppressed endogenous cholesterol synthesis (Fig. 7B) as a function of the concentration of lipoprotein cholesterol in the incubation medium. Normal HDL had no effect on these parameters. The down-regulation of LDL receptor activity that occurs when cells are incubated with normal LDL was paralleled when the cholesterol source was patient HDL (Fig. 7C).

TABLE 2. Volume of medium cleared of [^{14}C]sucrose in the presence of various lipoproteins during incubation with human skin fibroblasts[a]

Addition to Medium (Concentration)	[^{14}C]Sucrose Cleared
	μl medium/mg cell protein
[^{14}C]Sucrose (2.3 nmol/ml)	3.01
[^{14}C]Sucrose (2.3 nmol/ml)	
plus normal LDL (5 μg/ml)	2.93
plus normal LDL (50 μg/ml)	2.95
plus normal HDL (5 μg/ml)	2.98
plus normal HDL (50 μg/ml)	3.05
plus patient HDL (5 μg/ml)	2.99
plus patient HDL (50 μg/ml)	3.02

[a] Cell monolayers were incubated in medium containing 10% LPDS for 24 hr before addition of the indicated final concentrations of D-[U-^{14}C]sucrose (434 mCi/mmol) and lipoproteins. After 4 hr further incubation at 37°C, the cells were harvested for measurement of [^{14}C]sucrose uptake as described under Materials and Methods. Each result is the mean of duplicate dishes. Patient HDL was isolated from a patient (#1) with primary biliary cirrhosis; it reduced LDL binding to 16% of control values under the conditions shown in Fig. 2 and had a cholesterol/phospholipid molar ratio of 0.62 compared to 0.22 for normal HDL.

TABLE 3. Ability of HDL from a patient with alcoholic cirrhosis to increase the cholesteryl ester content of cultured human skin fibroblasts[a]

Added Lipoprotein	Cell Cholesterol	Cell Cholesteryl Ester
	μg/mg cell protein	
None	37.3 ± 2.1	1.6 ± 0.5
Normal LDL	47.6 ± 9.9	16.1 ± 2.8[c]
Normal HDL	32.5 ± 2.9[b]	1.1 ± 0.2
Patient HDL	41.0 ± 6.6	6.0 ± 1.0[c]

[a] Cell monolayers were incubated in medium containing 10% LPDS for 24 hr before addition of lipoproteins (final concentration 25 μg total cholesterol/ml culture medium). After 24 hr further incubation, the cells were washed and collected for measurement of their cholesterol and cholesteryl ester contents by gas–liquid chromatography. Results are expressed as the mean ± SD of values obtained from four individual 100-mm-diameter dishes. The HDL from a patient (# 7) with alcoholic cirrhosis reduced LDL binding to 26% of control values under the conditions shown in Fig. 2, and contained 0.66 mg of total cholesterol per mg protein (19.8% as ester). Normal LDL and normal HDL contained 1.82 mg (73.0% as ester) and 0.29 mg (86.1% as ester) total cholesterol per mg protein, respectively.

[b] $P < 0.05$.

[c] $P < 0.001$.

DISCUSSION

The present study has established that the HDL of patients with liver disease can markedly inhibit the binding, internalization, and degradation of LDL by cultured human skin fibroblasts. By contrast, HDL from normal subjects had only a small inhibitory effect on these processes, a finding in agreement with other workers (27, 30). Patient HDL was also inhibitory at 4°C, when cellular uptake of lipoproteins does not occur (22), suggesting that reduced internalization and degradation of LDL at 37°C were a consequence of impaired binding. Partially delipidated HDL was inhibitory; but no inhibition occurred when patient HDL was treated with cyclohexanedione to modify apolipoprotein arginyl residues. These observations suggest that apolipoproteins

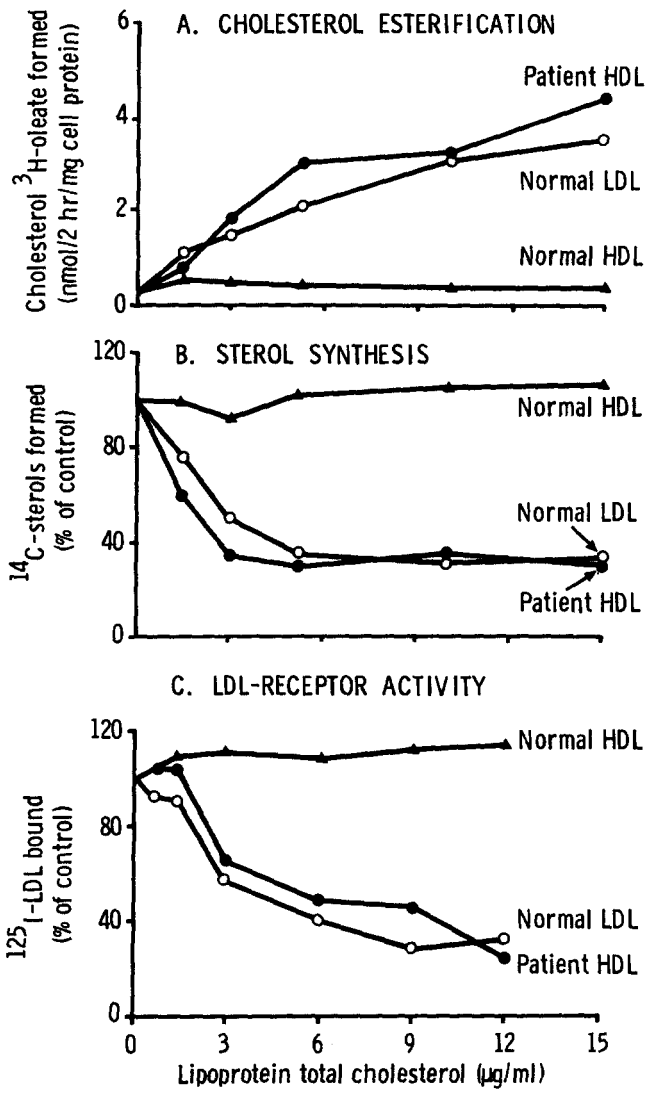

Fig. 7. Ability of liver disease HDL to stimulate cholesterol esterification and suppress sterol synthesis and LDL-receptor activity in cultured skin fibroblasts. Cell monolayers were preincubated with medium containing 10% LPDS for 24 hr, followed by a further 24 hr at 37°C with HDL from a patient (# 1) with primary biliary cirrhosis (●), normal HDL (▲), or normal LDL (○) added at equal total cholesterol concentrations. The patient HDL reduced ^{125}I-labeled LDL binding to 16% of control values (Table 1); following delipidation, it contained 6.7% of apoE and 6.7% of the unidentified apolipoproteins. Each point is the mean of duplicate dishes. Following incubation with the test lipoproteins: panel A, [^{3}H]oleic acid was added (0.1 mM, 52 dpm/pmol) and the cells were collected 2 hr later for measurement of ^{3}H incorporated into cholesteryl esters. Panel B, [^{14}C]acetate was added (0.1 mM, 9.5 mCi/mmol) and the cells were harvested 2 hr later to measure ^{14}C incorporation into nonsaponifiable lipids. The results are expressed as a percentage of ^{14}C-labeled sterols formed in control dishes (345 pmol ^{14}C-labeled sterols formed per hr per mg cell protein). Panel C, the cell monolayers were washed and then incubated 2 hr at 4°C with 7.5 μg protein/ml ^{125}I-labeled LDL (293 cpm/ng protein). Results are expressed as the percentage of control dishes (64 ng of ^{125}I-labeled LDL protein bound per mg cell protein).

of patient HDL are bound by the same high affinity cell-surface receptors and so interfere with binding of LDL.

Our patients with liver disease had abnormalities in their HDL apolipoprotein composition as judged by polyacrylamide gel electrophoresis and isoelectric focusing. Their mean apoE content was increased and significantly correlated with the ability of the HDL to inhibit LDL binding. Enrichment in apoE of the HDL fraction of patients with alcoholic hepatitis has previously been reported by several groups (8, 9, 31, 32). Our findings suggest that this may be a common feature of human liver disease; we found that patients with obstructive jaundice, as well as patients with parenchymal liver disease of varying etiology and severity, also have apoE-rich HDL. Presumably, apoE accumulates in the HDL fraction as a consequence of plasma lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43) deficiency (11, 12), a key enzyme in lipoprotein metabolism which is synthesized mainly in the liver (33). In addition to high apoE levels, Tada, Fidge, and Nestel (31) have found two abnormal apoE complexes in the HDL of patients with severe alcoholic hepatitis; one was identified as the apoE-apoA-II complex, previously described in the HDL of some normal subjects (5), while the other appeared to be an apoE trimer. We, and others (8), have not been able to detect these complexes in the HDL of jaundiced patients, possibly because their appearance is related to the severity of the liver disease. Unexpectedly, isoelectric focusing revealed a further abnormality in inhibitory patient HDL; it invariably contained two additional apolipoproteins not seen in normal HDL and their presence also correlated with the ability of the HDL to compete with LDL for binding sites. The significance of this finding remains to be established.

When inhibitory patient HDL was labeled with ^{125}I it was bound by the cultured fibroblasts in a saturable manner, similar to that seen with normal LDL. This binding was inhibited by LDL, supporting the previous conclusion that patient HDL and LDL were bound by the same sites. Following binding, patient ^{125}I-labeled HDL seemed to be taken up and degraded, suggesting that it may also deliver cholesterol to the cells. This was confirmed by incubation of patient HDL with fibroblasts; the rate of intracellular cholesterol esterification rose and the content of cholesteryl ester in the cells increased. Patient HDL was also effective at suppressing endogenous cholesterol synthesis and reducing the activity of LDL receptors. One possible explanation for these effects of patient HDL on fibroblast cholesterol metabolism is that they are secondary to changes in lipid composition of the cell surface membrane. In severe liver disease the lipoproteins, including HDL (see footnote to Table 2), are enriched in cholesterol as a consequence of plasma LCAT deficiency (11, 12). Incubation of cultured fibroblasts and other cells with such cholesterol-rich lipoproteins raises the cholesterol/phospholipid molar ratio of their membranes (34) and reduces membrane fluidity

(35). Change in cell-surface membrane fluidity is known to alter the capability of cells for endocytosis (36). However, we found no evidence for enhanced uptake of patient HDL by nonadsorptive pinocytosis; clearance of sucrose from incubation medium containing patient HDL was 100-fold less than that of patient HDL itself.

These effects of apoE-enriched patient HDL, therefore, supported our hypothesis that patient HDL would be taken up through the LDL-receptor pathway and would regulate cellular cholesterol metabolism. Mahley and co-workers (37) first established that the LDL-receptor was not specific for apoB; HDL_c, a cholesteryl ester-rich particle induced by cholesterol feeding in pigs and dogs and lacking apoB, was also bound by the LDL-receptor. Binding was attributed to the high apoE content of HDL_c and subsequent studies showed that the E apolipoprotein had up to 100-fold greater binding affinity for the cell-surface receptor than the apoB of LDL (16). The apoE in normal human HDL is largely confined to HDL_1 (38), a subclass present in low concentration which increases when the cholesterol content of the diet is raised (28, 35). There is evidence that HDL_1, like LDL, is taken up by cultured fibroblasts and after degradation affects cell cholesterol metabolism (6). More recently an apoE-rich HDL subfraction from a patient with familial LCAT deficiency has been shown to be internalized and degraded by cultured fibroblasts (39), while a fraction of HDL from patients with abetalipoproteinemia enriched in apoE suppressed cholesterol synthesis in cultured cells (40).

In the present study we have not attempted to determine whether the effects of our patient HDL are confined to a particular subfraction, nor whether binding activity resides in the two unusual apolipoproteins as well as in apoE. Normal human plasma HDL is heterogeneous; although it is usually fractionated by preparative ultracentrifugation into two major subclasses, HDL_{2b} (1.063–1.10 g/ml) and HDL_3 (1.125–1.21 g/ml), minor fractions are also recognized (32, 33, 38). In liver disease even greater heterogeneity is seen (8, 11, 12). The HDL in some of our patients contained "stacked discs" by electron microscopy which were present in variable amounts, presumably because of differences in the severity of the liver disease (11, 12). These discs are thought to represent newly synthesized (nascent) HDL secreted into the splanchnic bed and/or to be derived from redundant surface components of triglyceride-rich lipoproteins (33); they are usually rapidly converted to spherical HDL particles in the plasma by action of LCAT. There was no obvious correlation between the presence of stacked discs and the ability of the total patient HDL to compete with LDL for binding sites, perhaps because the apoE in discoidal particles has a different conformation and poorer binding ability than that in spherical HDL. Techniques to separate the components of the heterogeneous HDL fraction in primary and secondary LCAT deficiency are becoming more readily available (8, 39, 41). In future studies it should therefore be possible to establish whether uptake of our patient HDL is indeed restricted to particular subfractions, as suggested by experiments with other abnormal HDL fractions (39, 40) and by the marked differences in apoE content across the HDL density range reported in alcoholic hepatitis (8). Presumably, inhibition of LDL uptake by such patient HDL subfractions will be more striking than that suggested by our findings with whole patient HDL.

The pathophysiological significance of our findings is uncertain, in part reflecting our ignorance of lipoprotein metabolism in normal subjects and in patients with liver disease. One conclusion is that LDL catabolism by receptor-mediated pathways may be impaired in vivo in liver disease as a consequence of competition by abnormal HDL. But direct measurements of receptor-mediated LDL clearance in normal man are conflicting; some studies suggest only one-third of LDL is catabolized through the receptor pathway (42) while others report as much as 80% (43). There is also controversy over the relative contributions of the liver and peripheral tissues to LDL catabolism (44), and whether the liver in adult man has functional LDL receptors (45, 46). Finally, the role of apoE-rich HDL_1 particles is uncertain; potentially they may be taken up by peripheral cells as a source of cholesterol but, alternatively, HDL_1 (or its precursor) may be involved in cholesterol removal from peripheral cells for delivery to the liver via the apoE-specific receptors on hepatocyte surfaces (46, 47). We have previously shown in patients with parenchymal liver disease that mean plasma LDL concentrations are normal even though levels of the precursor, very low density lipoprotein, are markedly reduced (11). This could be explained by decreased LDL catabolism and is supported by our finding of a decreased fractional catabolic rate of LDL in such patients (a mean of 26%/24 hr in six patients compared to 38%/24 hr in eight normal subjects, $P < 0.01$) (R. C. Day, unpublished experiments.). Impaired LDL clearance might be explained by competition between abnormal HDL and LDL for binding by receptors, whether on adult liver (45) or on peripheral tissue (2). Alternatively, impaired LDL catabolism in parenchymal liver disease could be due to reduced hepatic uptake, either by receptor-dependent or receptor-independent pathways, secondary to liver damage.

We thank Professor Dame Sheila Sherlock for allowing us to study patients under her care. J.S.O. thanks the Wellcome Trust for an Interdisciplinary Research Fellowship and the British Heart Foundation for a project grant. H.G. was a British Heart Foundation Junior Research Fellow and R.C.D. was supported by a research scholarship from the Prophit Fund.

Manuscript received 11 July 1983.

REFERENCES

1. Owen, J. S., P. Mistry, H. Goodall, P. Chu, D. S. Harry, R. C. Day, and N. McIntyre. 1980. Low density lipoprotein (LDL) binding by cultured human skin fibroblasts: the inhibitory effect of high density lipoprotein (HDL) from patients with liver disease. *Gastroenterology.* **79:** 1119 (Abstract).
2. Brown, M. S., P. T. Kovanen, and J. L. Goldstein. 1981. Regulation of plasma cholesterol by lipoprotein receptors. *Science.* **212:** 628–635.
3. Goldstein, J. L., R. G. W. Anderson, and M. S. Brown. 1979. Coated pits, coated vesicles and receptor-mediated endocytosis. *Nature.* **279:** 679–685.
4. Innerarity, T. L., and R. W. Mahley. 1978. Enhanced binding by cultured human fibroblasts of apoE-containing lipoproteins as compared with low density lipoproteins. *Biochemistry.* **17:** 1440–1447.
5. Pitas, R. E., T. L. Innerarity, K. S. Arnold, and R. W. Mahley. 1979. Rate and equilibrium constants for binding of apoE HDL_c (a cholesterol-induced lipoprotein) and low density lipoproteins to human fibroblasts: evidence for multiple receptor binding to apoE HDL_c. *Proc. Natl. Acad. Sci. USA.* **76:** 2311–2315.
6. Innerarity, T. L., R. W. Mahley, K. H. Weisgraber, and T. P. Bersot. 1978. Apoprotein (E-A-II) complex of human plasma lipoproteins. II. Receptor binding activity of a high density lipoprotein subfraction modulated by the apo(E-A-II) complex. *J. Biol. Chem.* **253:** 6289–6295.
7. Florén, C-H., J. J. Albers, B. J. Kudchodkar, and E. L. Bierman. 1981. Receptor-dependent uptake of human chylomicron remnants by cultured skin fibroblasts. *J. Biol. Chem.* **256:** 425–433.
8. Weidman, S. W., J. B. Ragland, and S. M. Sabesin. 1982. Plasma lipoprotein composition in alcoholic hepatitis: accumulation of apolipoprotein E-rich high density lipoprotein and preferential reappearance of 'light'-HDL during partial recovery. *J. Lipid Res.* **23:** 556–569.
9. Ragland, J. B., P. Bertram, and S. M. Sabesin. 1978. Identification of nascent high density lipoproteins containing arginine-rich protein in human plasma. *Biochem. Biophys. Res. Commun.* **80:** 81–88.
10. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
11. Agorastos, J., C. Fox, D. S. Harry, and N. McIntyre. 1978. Lecithin-cholesterol acyltransferase and the lipoprotein abnormalities of obstructive jaundice. *Clin. Sci. Molec. Med.* **54:** 369–379.
12. Day, R. C., D. S. Harry, J. S. Owen, A. Y. Foo, and N. McIntyre. 1979. Lecithin-cholesterol acyltransferase and the lipoprotein abnormalities of parenchymal liver disease. *Clin. Sci.* **56:** 575–583.
13. Gidez, L. I., J. B. Swaney, and S. Murnane. 1977. Analysis of rat serum apolipoproteins by isoelectric focusing. I. Studies on the middle molecular weight subunits. *J. Lipid Res.* **18:** 59–68.
14. Kane, J. P. 1973. A rapid electrophoretic technique for identification of subunit species of apoproteins in serum lipoproteins. *Anal. Biochem.* **53:** 350–364.
15. Mahley, R. W., T. L. Innerarity, R. E. Pitas, K. H. Weisgraber, J. H. Brown, and E. Gross. 1977. Inhibition of lipoprotein binding to cell surface receptors of fibroblasts following selective modification of arginyl residues in arginine-rich and B apoproteins. *J. Biol. Chem.* **252:** 7279–7287.
16. McFarlane, A. S. 1958. Efficient trace-labeling of proteins with iodine. *Nature.* **182:** 53.
17. Bolton, A. E., and W. M. Hunter. 1973. The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. Application to the radioimmunoassay. *Biochem. J.* **133:** 529–539.
18. Innerarity, T. L., R. E. Pitas, and R. W. Mahley. 1979. Binding of arginine-rich (E) apoprotein after recombination with phospholipid vesicles to the low density lipoprotein receptors of fibroblasts. *J. Biol. Chem.* **254:** 4186–4190.
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
20. Goldstein, J. L., and M. S. Brown. 1974. Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J. Biol. Chem.* **249:** 5153–5162.
21. Mahley, R. W., and T. L. Innerarity. 1977. Interaction of canine and swine lipoproteins with the low density lipoprotein receptor of fibroblasts as correlated with heparin/manganese precipitability. *J. Biol. Chem.* **252:** 3980–3986.
22. Goldstein, J. L., S. K. Basu, G. Y. Brunschede, and M. S. Brown. 1976. Release of low density lipoprotein from its cell surface receptor by sulfated glycosaminoglycans. *Cell.* **7:** 85–95.
23. Tauber, J-P., D. Goldminz, I. Vlodavsky, and D. Gospodarowicz. 1981. The interaction of the high-density lipoprotein with cultured cells of bovine vascular endothelium. *Eur. J. Biochem.* **119:** 317–325.
24. Miller, N. E., D. B. Weinstein, and D. Steinberg. 1977. Binding, internalization, and degradation of high density lipoprotein by cultured normal human fibroblasts. *J. Lipid Res.* **18:** 438–450.
25. Owen, J. S., J. S. Medeiros, V. Ramalho, and Y. Cechinel. 1978. Determination of the proportion of cholesteryl ester in plasma. *Ann. Clin. Biochem.* **15:** 226–227.
26. Stokke, K. T., and K. R. Norum. 1971. Determination of lecithin:cholesterol acyltransferase in human blood plasma. *Scand. J. Clin. Lab. Invest.* **27:** 21–27.
27. Miller, N. E., D. B. Weinstein, T. E. Carew, T. Koschinsky, and D. Steinberg. 1977. Interaction between high density and low density lipoproteins during uptake and degradation by cultured human fibroblasts. *J. Clin. Invest.* **60:** 78–88.
28. Rose, H. G., and M. Oklander. 1965. Improved procedure for the extraction of lipids from human erythrocytes. *J. Lipid Res.* **6:** 428–431.
29. Stein, O., D. B. Weinstein, Y. Stein, and D. Steinberg. 1976. Binding, internalization, and degradation of low density lipoprotein by normal human fibroblasts and by fibroblasts from a case of homozygous familial hypercholesterolemia. *Proc. Natl. Acad. Sci. USA.* **73:** 14–18.
30. Mahley, R. W., T. P. Bersot, T. L. Innerarity, A. Lipson, and S. Margolis. 1978. Alterations in human high-density lipoproteins, with or without increased plasma-cholesterol, induced by diets high in cholesterol. *Lancet.* **II:** 807–809.
31. Tada, N., N. Fidge, and P. Nestel. 1979. Identification and characterisation of mixed disulphide complexes of E apoprotein in high density lipoproteins of subjects with

acute alcoholic hepatitis. *Biochem. Biophys. Res. Commun.* **90:** 297–304.
32. Marcel, Y. L., C. Vezina, D. Emond, and G. Suzue. 1980. Heterogeneity of human high density lipoprotein: presence of lipoproteins with and without apoE and their roles as substrates for lecithin:cholesterol acyltransferase reaction. *Proc. Natl. Acad. Sci. USA.* **77:** 2969–2973.
33. Owen, J. S., and N. McIntyre. 1982. Plasma lipoproteins and lipid transport. *Trends Biochem. Sci.* **7:** 95–98.
34. Owen, J. S., and M. P. T. Gillett. 1983. Plasma lipids, lipoproteins and cell membranes. *Biochem. Soc. Trans.* **11:** 336–339.
35. Owen, J. S., K. R. Bruckdorfer, R. C. Day, and N. McIntyre. 1982. Decreased erythrocyte membrane fluidity and altered lipid composition in human liver disease. *J. Lipid Res.* **23:** 124–132.
36. Heiniger, H-J., A. A. Kandutsch, and H. W. Chen. 1976. Depletion of L-cell sterol depresses endocytosis. *Nature.* **263:** 515–517.
37. Mahley, R. W., K. H. Weisgraber, T. P. Bersot, and T. L. Innerarity. 1978. Effects of cholesterol feeding on human and animal high density lipoproteins. *In* High Density Lipoproteins and Atherosclerosis. A. M. Gotto, Jr., N. E. Miller, and M. F. Oliver, editors. Elsevier, North-Holland, Amsterdam. 149–176.
38. Weisgraber, K. H., and R. W. Mahley. 1980. Subfractionation of human high density lipoproteins by heparin-Sepharose affinity chromatography. *J. Lipid Res.* **21:** 316–325.
39. Soutar, A. K., B. L. Knight, and N. B. Myant. 1982. The characterization of lipoproteins in the high density fraction obtained from patients with familial lecithin:cholesterol acyltransferase deficiency and their interaction with cultured human fibroblasts. *J. Lipid Res.* **23:** 380–390.
40. Blum, C. B., R. J. Decklebaum, L. D. White, A. R. Tall, and J. Cornicelli. 1982. Role of apolipoprotein E-containing lipoproteins in abetalipoproteinemia. *J. Clin. Invest.* **70:** 1157–1169.
41. Mitchell, C. D., W. C. King, K. R. Applegate, T. Forte, J. A. Glomset, K. R. Norum, and E. Gjone. 1980. Characterization of apolipoprotein E-rich high density lipoproteins in familial lecithin:cholesterol acyltransferase deficiency. *J. Lipid Res.* **21:** 625–634.
42. Shepherd, J., S. Bicker, A. R. Lorimer, and C. J. Packard. 1979. Receptor-mediated low density lipoprotein catabolism in man. *J. Lipid Res.* **20:** 999–1006.
43. Kesäniemi, Y. A., J. L. Witzum, and U. P. Steinbrecher. 1983. Receptor-mediated catabolism of low density lipoprotein in man. *J. Clin. Invest.* **71:** 950–959.
44. Attie, A. D., R. C. Pittman, and D. Steinberg. 1982. Hepatic catabolism of low density lipoprotein: mechanisms and metabolic consequences. *Hepatology.* **2:** 269–281.
45. Harders-Spengel, K., C. B. Wood, G. R. Thompson, N. B. Myant, and A. K. Soutar. 1982. Difference in saturable binding of low density lipoprotein to liver membranes from normocholesterolemic subjects and patients with heterozygous familial hypercholesterolemia. *Proc. Natl. Acad. Sci. USA.* **79:** 6355–6359.
46. Mahley, R. W., D. Y. Hui, T. L. Innerarity, and K. H. Weisgraber. 1981. Two independent lipoprotein receptors on hepatic membranes of dog, swine and man. *J. Clin. Invest.* **68:** 1197–1206.
47. Hui, D. Y., T. L. Innerarity, and R. W. Mahley. 1981. Lipoprotein binding to canine hepatic membranes. Metabolically distinct apoE and apoB,E receptors. *J. Biol. Chem.* **256:** 5646–5655.