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Research Article

High density lipoprotein (HDL) inhibited the binding (trypsin-releasable radioactivity), internalization (cell-associated radioactivity after trypsinization), and degradation (TCA-soluble non-iodide radioactivity) of ^{125}I -low density lipoprotein (^{125}I -LDL) by cultured normal human fibroblasts. At HDL:LDL molar ratios of 25:1 (protein ratios about 5:1), these parameters were reduced by about 25%. Unlabeled LDL was about 25 times more effective in reducing ^{125}I -LDL binding, implying that if HDL and LDL bind at common sites the affinity of HDL for these sites is very low or that the interaction is on some other basis. The fractional reduction in ^{125}I -LDL binding at a given HDL: ^{125}I -LDL ratio was independent of ^{125}I -LDL concentration and occurred equally with fibroblasts from a subject with homozygous familial hypercholesterolemia. Reciprocally, the binding, internalization, and degradation of ^{125}I -HDL were reduced by LDL. Preincubation of fibroblasts with HDL (or LDL) reduced the subsequent binding of ^{125}I -LDL (or ^{125}I -HDL) during a second incubation. In other studies HDL reduced the net increase in cell cholesterol content induced by incubation with LDL. HDL alone had no net effect on cell cholesterol content.

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Interaction between High Density and Low Density Lipoproteins during Uptake and Degradation by Cultured Human Fibroblasts

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ABSTRACT High density lipoprotein (HDL) inhibited the binding (trypsin-releasable radioactivity), internalization (cell-associated radioactivity after trypsinization), and degradation (TCA-soluble non-iodide radioactivity) of ^{125}I -low density lipoprotein (^{125}I -LDL) by cultured normal human fibroblasts. At HDL:LDL molar ratios of 25:1 (protein ratios about 5:1), these parameters were reduced by about 25%. Unlabeled LDL was about 25 times more effective in reducing ^{125}I -LDL binding, implying that if HDL and LDL bind at common sites the affinity of HDL for these sites is very low or that the interaction is on some other basis. The fractional reduction in ^{125}I -LDL binding at a given HDL: ^{125}I -LDL ratio was independent of ^{125}I -LDL concentration and occurred equally with fibroblasts from a subject with homozygous familial hypercholesterolemia. Reciprocally, the binding, internalization, and degradation of ^{125}I -HDL were reduced by LDL. Preincubation of fibroblasts with HDL (or LDL) reduced the subsequent binding of ^{125}I -LDL (or ^{125}I -HDL) during a second incubation. In other studies HDL reduced the net increase in cell cholesterol content induced by incubation with LDL. HDL alone had no net effect on cell cholesterol content.

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These findings suggest that HDL reduces both the high affinity and the low affinity binding of LDL to human fibroblasts and that this in turn reduces the internalization and degradation of LDL. The effect of HDL on the LDL-induced changes in cell cholesterol content could be in part on this basis and in part on the basis of an HDL-stimulated release of cholesterol from the cells. These effects of HDL in vitro may be relevant to the negative correlations reported from in vivo studies between plasma HDL concentration and both body cholesterol pool size and the prevalence of clinically manifest atherosclerosis but further studies will be needed to establish this.

INTRODUCTION

A variety of peripheral cell types in culture, including fibroblasts (2, 3), smooth muscle cells (4, 5), and vascular endothelium (6), have been shown to take up and degrade plasma low density lipoprotein (LDL).¹ In fibroblasts this involves the binding of LDL to the cell surface, followed by its internalization and uptake into lysosomes (3, 7, 8). Here the protein component is degraded to TCA-soluble fragments, which rapidly leave the cell (2, 3). The cholesteryl ester of the lipoprotein is hydrolyzed (9) and the free cholesterol so released inhibits endogenous cholesterol synthesis through suppression of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (10). With continued exposure of the cells to high LDL levels, the number of cell surface binding sites for LDL is reduced, and this in turn reduces the rates of LDL inter-

¹Abbreviations used in this paper: HDL, high density lipoprotein; HFH, homozygous familial hypercholesterolemia; LDL, low density lipoprotein; LDS, lipoprotein-deficient fetal calf serum; PBS, Dulbecco's phosphate-buffered saline.

nalization and degradation (11). The importance of peripheral tissues in LDL metabolism *in vivo* has been suggested: (a) by the finding in experimental animals that the rate of removal of LDL from plasma is not reduced following total hepatectomy (12); and (b) by the finding that fibroblasts cultured from subjects with familial hypercholesterolemia do not internalize or degrade LDL at a normal rate (2, 3).

It appears, therefore, that the metabolism of LDL may involve a considerable flux of LDL cholesterol into peripheral tissues. Despite this, the cholesterol content of extrahepatic tissues, measured directly in biopsies (13, 14) or indirectly by isotope dilution analysis (15–17), has been only weakly correlated with the plasma total and LDL cholesterol concentrations. On the other hand, a recent study of hyperlipidemic subjects established that body cholesterol pool size shows a strong negative correlation with the plasma high density lipoprotein (HDL) concentration (17). Theoretically this could reflect a function of HDL in the removal of cholesterol from tissues, an inhibitory effect of HDL on cell cholesterol synthesis, an inhibition by HDL of the cellular uptake of LDL, or a combination of these mechanisms.

Support for a role of HDL in cholesterol transport from tissues, as originally proposed by Glomset (18), has been provided in studies of ascites tumor cells and cultured rat aortic smooth muscle cells (19). Other investigations have shown that HDL binds to cultured human fibroblasts (1) and swine aortic smooth muscle cells (20) but, in contrast to LDL, is only slowly internalized and degraded. In aortic smooth muscle cell cultures HDL was found to reduce the binding, internalization, and degradation of LDL (20). In the present study the possibility that the surface binding of HDL might interfere with the cellular uptake and degradation of LDL by human fibroblasts has been investigated.

METHODS

Materials. ^{125}I -Sodium iodide (carrier-free in 0.05 N NaOH) was obtained from Schwarz/Mann (Div. Becton, Dickinson & Co., Orangeburg, N. Y.), ^{131}I (25 mCi/ μg in NaOH) from Amersham/Searle Corp. (Arlington Hgts., Ill.), $[1\text{-}^{14}\text{C}]$ acetate (sodium salt; 58 mCi/mmol) from Dhom Products Ltd. (North Hollywood, Calif.), and D- $[^{14}\text{C}(U)]$ -sucrose (440–455 mCi/mmol) from New England Nuclear (Boston, Mass.). Cholesterol (recrystallized twice from ethyl acetate) and 5- α -cholestane were obtained from Applied Science Labs, Inc. (State College, Pa.), and human albumin and human γ -globulin from Sigma Chemical Co., (St. Louis, Mo.). Fetal calf serum was purchased from Irvine Scientific Sales Company (Irvine, Calif.). Dulbecco's modification of Eagle's minimal essential medium (21) and Dulbecco's phosphate-buffered saline (PBS; 22) were obtained from Gibco Diagnostics (Chagrin Falls, Ohio).

Cell cultures. Skin fibroblasts were grown in monolayer from a preputial biopsy of a normal infant and from a skin

biopsy of a 19-yr-old female with homozygous familial hypercholesterolemia (HFH; subject P₄ in reference 23; subject 2 in reference 24). Comparative studies of the metabolism of LDL and HDL by these cell lines have already been reported (1, 3). Cultures were maintained in a humidified incubator (95% air, 5% CO₂) at 37°C in Dulbecco's modification of Eagle's minimal essential medium containing 10% (vol/vol) fetal calf serum (final protein concentration, 4 mg/ml). Cells were studied between the 9th and 20th passages. Polystyrene tissue culture dishes (60 × 15 mm; Corning Glass Works, Corning, N. Y.) were seeded with 1–2 × 10⁵ cells and used 4–6 days later, at which time the cultures were 50–95% confluent. Growth curves had previously shown that the normal cell line reached a plateau at 1–2 × 10⁶ cells (approximately 600 μg protein) in 6–8 days (3). The mutant cells were fewer in number at confluency (6–8 × 10⁵ cells/dish). At the time of study, cell protein per dish was 300–600 μg for normal cells and 150–350 μg for mutant cells.

Lipoproteins and lipoprotein-deficient serum. Lipoproteins in the density ranges 1.020–1.055 g/ml (LDL) and 1.090–1.21 (HDL) were isolated from pooled fasting human plasma (normolipidemic subjects only) by preparative ultracentrifugation (25). The serum was adjusted to *d* 1.020 and centrifuged 24 h at 27,000 rpm in a Beckman-Spinco L2-65B centrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) using an SW27 rotor. The bottom fraction was recentrifuged at 1.020 to completely remove very low density lipoprotein and very low density lipoprotein remnants. The bottom fraction was then adjusted to *d* 1.09 and centrifuged 20–22 h at 50,000 rpm in a Beckman-Spinco 60 Ti rotor. The top fraction was adjusted to *d* 1.055, centrifuged as before, and the top fraction recentrifuged at *d* 1.055 to yield a washed *d* 1.020–1.055 fraction (LDL). The *d* > 1.090 fraction was recentrifuged at *d* 1.090 to remove any contaminating LDL and the bottom fraction was finally centrifuged twice at *d* 1.21 to yield the washed *d* 1.090–1.21 fraction (HDL).

Isolated fractions were dialyzed against buffer containing 20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, and 0.3 mM EDTA, sterilized by passage through a Millipore filter (0.22 or 0.45 μm /Millipore Corp., Bedford, Mass.), and stored at 4°C. Protein content was determined according to Lowry et al. (26). Lipoprotein fractions were shown to be free of cross-contamination with other lipoprotein classes by immunodiffusion against specific rabbit antisera to human LDL and HDL (27). The sensitivity of the method, tested with serial dilutions of LDL, would have allowed the detection of as little as 1% LDL contamination of the HDL fraction. The B apoprotein content of the HDL fraction, measured for us by Dr. John J. Albers by radioimmunoassay, was <0.5% of the total protein in the fraction.

Lipoprotein-deficient fetal calf serum (LDS) was prepared as previously described (5). The final cholesterol content, determined by gas-liquid chromatography as described below, was <2.5 $\mu\text{g}/\text{ml}$.

^{125}I -HDL, ^{125}I -LDL, and ^{131}I -HDL were prepared by a modification of the iodine monochloride method of McFarlane (28) as previously described (5). Specific activities were 198–561 cpm/ng protein for ^{125}I -HDL, 212–456 cpm/ng for ^{125}I -LDL, and 188 cpm/ng for ^{131}I -HDL. Less than 3% of radioactivity in the final preparations was TCA-soluble and <2% was extractable into chloroform:methanol (2:1 vol/vol). More than 93% of the radioactivity in ^{125}I -HDL was precipitable with rabbit antiserum to human HDL; none was precipitated by rabbit antiserum to human albumin. Column chromatography of delipidated, lyophilized ^{125}I -HDL apoproteins on Sephadex G-100 (29) showed that approximately 53% of the radioactivity was in apoprotein A1, 42% in apo-

protein A11, and 5% in C peptides. No ^{125}I -labeled lipoprotein was used more than 4 wk after its preparation.

The integrity of ^{125}I -HDL was assessed by diluting it up to 20-fold with unlabeled HDL and studying its metabolism by cells exposed to a constant total HDL concentration of 23 $\mu\text{g}/\text{ml}$. Binding, internalization, and degradation of the variously diluted ^{125}I -HDL by fibroblasts (determined as described below) decreased in direct proportion to the extent of dilution, suggesting that the cells did not distinguish between labeled and unlabeled HDL. Similar results have been reported previously for ^{125}I -LDL (3). Recentrifugation of ^{125}I -HDL and ^{125}I -LDL at densities of 1.21 and 1.063 g/ml , respectively, had no appreciable effect on the binding, internalization, or degradation of either lipoprotein.

Lipoprotein uptake and degradation. 18 h before an experiment the medium was removed from each dish and replaced with 3 ml of fresh medium containing 5% (vol/vol) of LDS. Immediately before the experiment this was replaced with 2 ml of fresh medium containing 5% LDS. Cells to be studied at 0°C were placed on crushed ice for 15 min before starting the experiment, and then held on ice in a 4°C cold room.

At the end of an incubation with ^{125}I - or ^{131}I -labeled lipoprotein, the medium was removed and an aliquot assayed for total radioactivity. Lipoprotein degradation was calculated from measurement of TCA-soluble ^{125}I in the medium after removal of ^{125}I -iodide (30). Net degradation was calculated as the difference between values obtained from identical incubations in the presence and in the absence of cells (2, 3). The amount of TCA-soluble, noniodide ^{125}I remaining with the cells after an 18-h incubation was $<2\%$ that accumulated in the medium in 18 h.

After removal of the medium, the cells were washed and harvested by trypsinization as previously described (3). The cells were sedimented by centrifugation at 4°C (3,000 g for 10 min), and an aliquot of the supernatant fraction was assayed for ^{125}I or ^{131}I radioactivity. The radioactivity released by trypsin was considered to represent lipoprotein bound to the cell surface (3, 4, 30). Release of radioactivity from dishes which had been incubated with ^{125}I -LDL or ^{125}I -HDL in the absence of cells was only 4–8% of that recovered from identical dishes containing cells, excluding any important release by trypsin of labeled lipoprotein adsorbed to the surface of the dish. The amount of radioactivity in the final PBS wash was shown to be $<4\%$ of that subsequently released by trypsin. Values for ^{125}I -HDL and ^{125}I -LDL binding were not reduced by the addition of albumin (2 mg/ml) to the fourth and fifth PBS washes.

The cells were washed by suspending them in 4 ml PBS and recentrifuging at 3,000 g for 20 min. The pellet was assayed for radioactivity, and then dissolved in 0.2 ml 1 N KOH (20°C , 24 h). The cell digest was diluted with water to 1 ml and aliquots were removed for protein assay (26) and for measurement of total, lipid-soluble (chloroform:methanol, 2:1 vol/vol) and TCA-soluble radioactivity. The non-lipid TCA-precipitable radioactivity remaining associated with the cells after trypsinization was considered to represent internalized lipoprotein (3, 4, 30).

Lipoprotein concentrations and data on binding, internalization, and degradation are expressed in terms of protein content, unless otherwise stated.

Cell cholesterol content. 18 h before an experiment, the medium was removed from each dish and replaced with 3 ml of medium containing 5% LDS. At the beginning of the experiment this was replaced with 2 ml of fresh medium containing 5% LDS with or without added lipoproteins, and dishes were incubated for 18 h. The medium was then re-

moved and the cells washed with 6×2 ml PBS before being harvested with a rubber policeman. The cells were pelleted by centrifugation (3,000 g , 10 min, 4°C) and dissolved in 0.2 ml 1 N KOH. The dissolved pellet was diluted to 1.0 ml and aliquots were removed for protein assay (26). After addition of 5 μg of 5- α -cholestane to serve as internal standard, the remainder was saponified (1 N KOH in 70% ethanol for 2–3 h at 60°C) and diluted with 1.0 ml water. The nonsaponifiable lipids were extracted with 4.2 ml hexane and the cholesterol content of the extract determined by gas-liquid chromatography (31).

^{14}C Sucrose uptake (fluid endocytosis). The uptake of radiolabeled sucrose by cultured fibroblasts has been shown to be a suitable marker for the quantitation of fluid uptake by pinocytosis (32, 33). 18 h before an experiment the medium was removed from each dish and replaced with medium containing 5% LDS. At the beginning of the experiment this was replaced with fresh medium containing 5% LDS and D- ^{14}C (U)sucrose at a concentration of 1.0–5.5 $\mu\text{mol}/\text{liter}$ (0.5–2.5 $\mu\text{Ci}/\text{ml}$). After further incubation for 18 h at 37°C , the medium was removed and an aliquot added to 15 ml of scintillation solution (10.5 ml toluene, 4.5 ml Triton, 60 mg 2,5-diphenyloxazole, 0.8 mg [1,4-bis-2-(5-phenyloxazolyl)-benzene]) for determination of ^{14}C radioactivity. The cells were washed 10 times with 3 ml PBS, harvested with a rubber policeman, and dissolved in 0.2 ml 1 N NaOH. Aliquots were taken for protein determination (26) and liquid scintillation counting. Counting efficiency, measured by internal standardization, averaged 84%. Clearance of [^{14}C]sucrose (μl medium/mg cell protein) was calculated by dividing the uptake of radioactivity (cpm/mg cell protein) by the concentration of radioactivity in the medium (cpm/ μl). [^{14}C]sucrose uptake was linear for at least 27 h and was unaffected by the addition of unlabeled sucrose (45 $\mu\text{mol}/\text{liter}$). The total radioactivity in the final PBS wash was $<3\%$ of that found to be associated with the cells. When cells that had been preincubated for 18 h with [^{14}C]sucrose were washed and incubated for 3 h in fresh sucrose-free medium, there was no significant release of radioactivity.

RESULTS

The effects of unlabeled HDL on the binding, internalization, and degradation of ^{125}I -LDL (4.9 $\mu\text{g}/\text{ml}$) by normal human fibroblasts are shown in Fig. 1. Addition of increasing amounts of HDL to the culture medium was associated first with a small (5–10%) but consistently observed rise in ^{125}I -LDL binding, internalization, and degradation, and then, at HDL: ^{125}I -LDL molar ratios² $>5:1$, with progressive reductions in each parameter. At a given molar ratio, the effects on binding, internalization, and degradation were of comparable magnitude, amounting to 25–50% at a molar ratio of 50:1. A shown in Fig. 1, the inhibition of ^{125}I -LDL binding, internalization, and degradation

² Molar lipoprotein concentrations were calculated assuming a molecular weight of 550,000 for LDL protein (34, 35) and a molecular weight of 110,000 for HDL protein (36, 37). Thus, a 5:1 molar ratio of HDL to LDL corresponds to a 1:1 protein concentration ratio. Human albumin: molecular weight, 69,000 (38). Human γ -globulin: molecular weight, 150,000 (38).

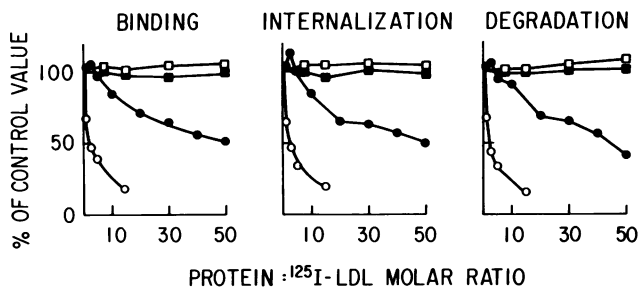


FIGURE 1 Effects of HDL, LDL, albumin, and γ -globulin on the binding, internalization, and degradation of ^{125}I -LDL by normal human fibroblasts. Cell monolayers were incubated for 18 h in medium containing 5% (vol/vol) LDS. This was then replaced with fresh medium containing 5% LDS, 4.9 $\mu\text{g}/\text{ml}$ of ^{125}I -LDL (specific activity, 414 cpm/ng), and either HDL (\bullet), LDL (\circ), human albumin (\blacksquare), or human γ -globulin (\square) at the indicated molar ratio to ^{125}I -LDL. After further incubation for 3 h at 37°C , the cells were harvested and the binding (left panel), internalization (middle panel), and degradation (right panel), of ^{125}I -LDL determined as described in Methods. Results are expressed as percentages of values obtained with medium containing only 5% LDS and 4.9 $\mu\text{g}/\text{ml}$ of ^{125}I -LDL (binding, 62 ng/mg; internalization, 439 ng/mg; degradation, 724 ng/mg). Each point represents the mean of duplicate determinations. 1 mg LDL protein \approx 1.8 nmol LDL; 1 mg HDL protein \approx 9.0 nmol HDL; 1 mg albumin \approx 14.5 nmol; 1 mg γ -globulin \approx 6.7 nmol (see Results).

by HDL was much less than that produced by the addition of similar molar quantities of unlabeled LDL. Human albumin and γ -globulin had no effect on ^{125}I -LDL uptake and degradation (Fig. 1).

The percentage reduction in the binding, internalization, and degradation of ^{125}I -LDL at a given HDL: ^{125}I -LDL ratio was independent of the ^{125}I -LDL concentration. This is illustrated in Fig. 2, which compares the effects of HDL on ^{125}I -LDL binding, internalization, and degradation by normal fibroblasts at ^{125}I -LDL concentrations of 5 $\mu\text{g}/\text{ml}$ and 104 $\mu\text{g}/\text{ml}$.

A similar effect of HDL on ^{125}I -LDL binding was observed with fibroblasts from a subject with HFH (Table I). Whereas the absolute values for ^{125}I -LDL binding were lower with HFH cells than with normal cells ($P < 0.001$), the percentage reduction in binding produced by HDL was similar with the two cell types.

The high affinity binding sites for LDL in normal fibroblasts are effectively destroyed by proteolytic enzymes (2). To further test whether the observed HDL-LDL interaction was limited to interaction at high affinity sites, cells were treated with pronase and the effects of HDL on ^{125}I -LDL binding were determined before and after such treatment. As shown in Table II, pronase treatment reduced LDL binding but the HDL effect on LDL binding persisted after pronase treatment. High affinity binding sites for LDL are also reduced in cells incubated with cholesterol or related sterols (11). As shown in Table II, the HDL

effect on ^{125}I -LDL binding was comparable after such an incubation with sterols.

Unlabeled LDL was found to alter the binding, internalization, and degradation of ^{125}I -HDL by normal fibroblasts. Inasmuch as the rate of degradation of HDL by human fibroblasts is very low relative to that of LDL (1), experiments with ^{125}I -HDL were performed in medium containing 2% LDS, rather than 5% LDS, to reduce the rate of release of free iodide and thereby obtain degradation values less influenced by background (no cell control) corrections. The results of one such experiment appear in Fig. 3. At LDL: ^{125}I -HDL molar ratios of $<2:1$ a 10–15% increase in ^{125}I -HDL degradation was sometimes observed. At molar ratios exceeding 2:1, however, the addition of increasing amounts of LDL to the medium was associated with progressive reductions in the bind-

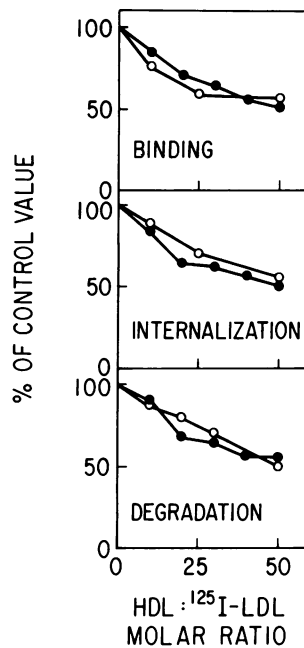


FIGURE 2 Effect of HDL on the binding, internalization, and degradation of ^{125}I -LDL by normal human fibroblasts at two widely different concentrations of ^{125}I -LDL. Cell monolayers were incubated for 18 h in medium containing 5% LDS. This was then replaced with fresh medium containing 5% LDS and either 5 $\mu\text{g}/\text{ml}$ (\bullet) or 104 $\mu\text{g}/\text{ml}$ (\circ) of ^{125}I -LDL (414 cpm/ng) and unlabeled HDL at the indicated molar ratios to ^{125}I -LDL. After further incubation for 3 h at 37°C the cells were harvested for measurement of ^{125}I -LDL binding, internalization, and degradation as described in Methods. Results are expressed as percentages of values obtained in the absence of HDL. (At 5 μg ^{125}I -LDL/ml: binding, 62 ng/mg; internalization, 439 ng/mg; degradation, 724 ng/mg. At 104 μg ^{125}I -LDL/ml: binding, 346 ng/mg; internalization, 1,258 ng/mg; degradation, 2,210 ng/mg). Each point represents the mean of duplicate determinations. Molar conversion factors appear in the legend to Fig. 1.

TABLE I
Effects of High Density Lipoprotein on the Binding of ¹²⁵I-LDL to Fibroblasts from Normal and Homozygous Familial Hypercholesterolemic (HFH) Subjects

| Fibroblasts | ¹²⁵ I-LDL binding | | | HDL effect | P |
|-------------|-------------------------------|--|-----|------------|---|
| | ¹²⁵ I-LDL, 5 μg/ml | ¹²⁵ I-LDL, 5 μg/ml plus unlabeled HDL, 49 μg/ml | | | |
| | | ng/mg cell protein | % | | |
| Normal | 42.0±1.7 (4) | 30.9±1.7 (4) | -26 | <0.005 | |
| HFH | 20.5±0.5 (4) | 15.0±0.6 (4) | -25 | <0.001 | |

Cell monolayers were incubated for 18 h in medium containing 5% LDS. This was then replaced with fresh medium containing 5% LDS and the indicated concentrations of ¹²⁵I-LDL (219 cpm/ng) and unlabeled HDL. After further incubation for 2 h at 0°C, the cells were harvested and ¹²⁵I-LDL binding was determined as described in Methods. Results are given as mean±SEM (number of replicate dishes). Probability values were obtained by *t* test.

ing, internalization, and degradation of ¹²⁵I-HDL. At a molar ratio of 10:1 the reduction of ¹²⁵I-HDL binding by LDL was 50–70%, actually exceeding that produced by a similar molar concentration of unlabeled HDL. Human albumin and γ-globulin had no effect on ¹²⁵I-HDL binding, internalization, or degradation.

The effect of LDL on ¹²⁵I-HDL binding was compared in normal and HFH mutant fibroblasts. In three separate experiments, using ¹²⁵I-HDL at 1, 2.5, and 50 μg/ml, LDL reduced HDL binding in both cell lines (Table III), the mean reduction being 30% in normal cells and 32% in HFH cells. In two other experiments using cells from another HFH patient (J.P.), ¹²⁵I-HDL binding was reduced in a similar fashion in the presence of 4:1 and 5:1 molar ratios of unlabeled LDL to ¹²⁵I-HDL (data not shown).

The preceding studies suggested that the reduction in ¹²⁵I-HDL binding at a given LDL:¹²⁵I-HDL molar ratio was greater than that in ¹²⁵I-LDL binding at the same HDL:¹²⁵I-LDL molar ratio. This was confirmed by the results of the experiment shown in Fig. 4 in which the effect of HDL on the binding of ¹²⁵I-LDL was directly compared with the reciprocal effect of LDL on the binding of ¹²⁵I-HDL, using similar molar concentrations of labeled and unlabeled lipoproteins.

The experiments described thus far examined HDL-LDL interactions when both lipoproteins were added simultaneously to the medium and the results could have reflected an interaction between the lipoproteins in solution rather than at the cell membrane. An attempt was made to distinguish between these possibilities by examining the effect of preincubating cells with unlabeled HDL or LDL on the subsequent

binding of ¹²⁵I-LDL or ¹²⁵I-HDL, respectively, during a second incubation. After preincubation of normal fibroblasts with 76 μg/ml of HDL for 1 h at 0°C (followed by washing with 6 × 2 ml of PBS), the binding of ¹²⁵I-LDL at a concentration of 5 μg/ml in a second incubation (80 min, 0°C) was 25.0±1.6 ng/mg cell protein (mean±SEM from four replicate dishes). In contrast, the binding of ¹²⁵I-LDL to fibroblasts which had been preincubated for 1 h at 0°C with lipoprotein-deficient medium was 32.3±1.9 ng/mg (*n* = 4; *P* < 0.03). Reciprocally, preincubation of fibroblasts with 232 μg/ml of LDL reduced the subsequent binding of ¹²⁵I-HDL, at a concentration of 5 μg/ml, from 7.4±0.7 ng/mg (*n* = 4) to 4.8±0.1 ng/mg (*n* = 4; *P* = 0.01).

Studies were made of the simultaneous changes occurring in the binding of ¹²⁵I-LDL and ¹³¹I-HDL to fibroblasts with alterations of their ratio in the medium (Fig. 5). Alterations in the ¹³¹I-HDL: ¹²⁵I-LDL ratio were associated with reciprocal changes

TABLE II
Effects of High Density Lipoproteins on the Binding of ¹²⁵I-LDL to Normal Fibroblasts before and after Treatment to Reduce High Affinity LDL Binding

| Before treatment of cells* | Lipoprotein concentration | | ¹²⁵ I-LDL binding | | |
|---|---------------------------|-------|------------------------------|----------------------------|------------|
| | ¹²⁵ I-LDL | HDL | ¹²⁵ I-LDL alone | ¹²⁵ I-LDL + HDL | HDL effect |
| | | | ng/mg cell protein | % | |
| None | | | 34.8 | 27.3 | -22 |
| Pronase treatment | 5 | 50 | | | |
| | | | 7.3 | 6.3 | -14 |
| None | 100 | 1,000 | 97.9 | 71.9 | -27 |
| Pronase treatment | 100 | 1,000 | | | |
| | | | 62.9 | 39.9 | -36 |
| None | 5 | 50 | 30.1 | 20.1 | -33 |
| Incubation with Δ ⁷ -cholesten-3β-ol and cholesterol | 5 | 50 | | | |
| | | | 20.6 | 15.1 | -27 |

Incubation with Δ⁷-cholesten-3β-ol: cells were incubated in medium containing 5% LDS for 18 h, and then for an additional 18 h in the presence or absence of Δ⁷-cholesten-3β-ol (7.5 μg/ml) and cholesterol (12.5 μg/ml) before measurement of ¹²⁵I-LDL binding.

* Pronase treatment: cells were incubated in medium containing 5% LDS for 18 h, washed twice with PBS, incubated in medium (without serum) with and without pronase (1.5 μg/ml) for 23 min at 37°C, and washed twice with PBS before measurement of ¹²⁵I-LDL binding.

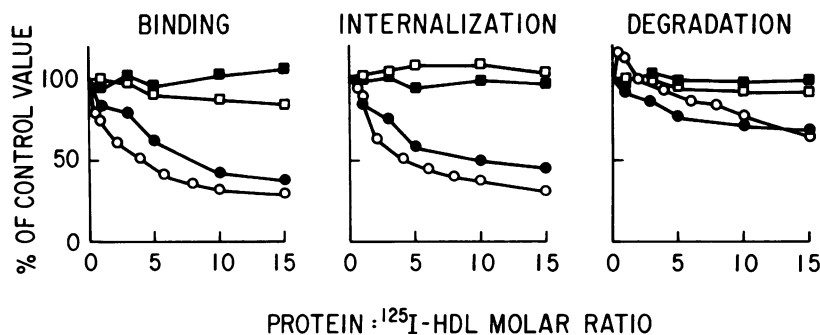


FIGURE 3 Effects of HDL, LDL, albumin, and γ -globulin on the binding, internalization, and degradation of ^{125}I -HDL by normal human fibroblasts. Cell monolayers were incubated for 18 h in medium containing 5% LDS. This was then replaced with similar medium containing 2% LDS, 5 $\mu\text{g}/\text{ml}$ of ^{125}I -HDL (specific activity, 198 cpm/ng), and either HDL (\bullet), LDL (\circ), human albumin (\blacksquare), or human γ -globulin (\square) at the indicated molar ratios to ^{125}I -HDL. After further incubation for 6 h at 37°C , the cells were harvested and the binding (left panel), internalization (middle panel), and degradation (right panel) of ^{125}I -HDL determined as described in Methods. Results are expressed as percentages of values obtained in the absence of added protein (binding, 31 ng/mg; internalization, 10.1 ng/mg; degradation, 34 ng/mg). Each point is the mean of duplicate determinations. Molar conversion factors appear in the legend to Fig. 1.

in the surface binding of the lipoproteins. The binding of ^{125}I -LDL at low concentrations was reduced on average by 1 molecule for every 20–25 molecules of ^{131}I -HDL bound. In the other experiment the binding of ^{131}I -HDL was reduced by 1 molecule for every 5–10 molecules of ^{125}I -LDL bound.

These data suggested that the binding of one lipoprotein to the cell surface interfered with the binding of the other. The associated reductions in lipoprotein internalization and degradation observed at 37°C could have been secondary to this phenomenon or alternatively could have reflected a more general inhibition of internalization mechanisms. The latter possibility was investigated by examining the effects of HDL and LDL on the cellular uptake of [^{14}C]-sucrose, which has been shown to be a suitable marker for the quantitation of fluid endocytosis (32, 33). The volume of medium cleared of [^{14}C]-sucrose by normal fibroblasts during an 18-h incubation of 37°C in lipoprotein-deficient medium averaged $3.40 \pm 0.29 \mu\text{l}/\text{mg}$ cell protein (mean \pm SD). This was unaffected by the addition of LDL or HDL to the medium at concentrations of up to 250 $\mu\text{g}/\text{ml}$ [^{14}C]-sucrose clearance 3.03–3.79 $\mu\text{l}/\text{mg}$).

Studies were also made of the effect of HDL on the changes normally induced by LDL in cell cholesterol content (Table IV). In accordance with previous reports (3, 5, 10, 39), incubation of normal fibroblasts with LDL, after prior incubation in lipoprotein-deficient medium, increased the cell content of cholesterol (+38%). In the presence of a very high concentration of HDL (molar ratio to LDL of approximately 60:1) the LDL-induced increase in cell cholesterol content was only 10% and this increase was not

statistically significant. In a second experiment (HDL at 1.043 $\mu\text{g}/\text{ml}$; LDL at 104 $\mu\text{g}/\text{ml}$), the LDL-induced increase in cell cholesterol content was 39% ($P < 0.025$) whereas the increase in the presence of HDL was only 11% and, again, not statistically significant. HDL alone at these high concentrations had no net effect on cell cholesterol content.

TABLE III
Effects of Low Density Lipoprotein on the Binding of ^{125}I -HDL to Normal Fibroblasts and Fibroblasts from a Patient with Homozygous Familial Hypercholesterolemia (HFH)

| Fibroblasts | Lipoprotein concentration | | ^{125}I -HDL binding | | |
|-------------|---------------------------|-------|-------------------------------|---------------------------------------|------------|
| | ^{125}I -HDL | LDL | ^{125}I -HDL alone | ^{125}I -HDL + unlabeled LDL | LDL effect |
| | $\mu\text{g}/\text{ml}$ | | ng/mg cell protein | | % |
| Normal | 1 | 200 | 1.60 | 1.25 | -22 |
| HFH | 1 | 200 | 2.00 | 1.45 | -27 |
| Normal | 2.5 | 50 | 7.30 | 5.75 | -21 |
| HFH | 2.5 | 50 | 12.05 | 5.25 | -56 |
| Normal | 50.0 | 1,000 | 28.1 | 14.6 | -48 |
| HFH | 50.0 | 1,000 | 46.5 | 35.0 | -24 |

In three separate experiments, cells were incubated in medium containing 5% LDS for 18 h at 37°C before assaying ^{125}I -HDL binding at 0°C for 2 h in the presence and absence of unlabeled LDL. Data represent means of duplicate cell dishes.

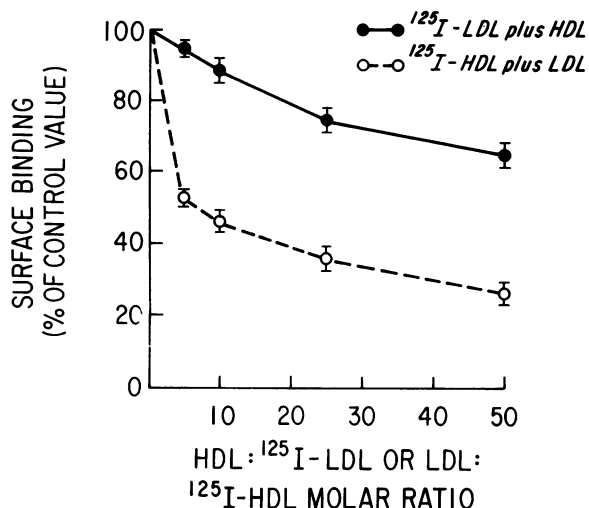


FIGURE 4 Relative changes in the binding of ¹²⁵I-LDL and ¹²⁵I-HDL to normal human fibroblasts in the presence of increasing concentrations of unlabeled HDL or LDL, respectively. Cell monolayers were incubated for 18 h in medium containing 5% LDS. This was then replaced with similar medium containing 10 μg/ml of ¹²⁵I-LDL and unlabeled HDL at the indicated molar ratio to ¹²⁵I-HDL (●), or 2 μg/ml ¹²⁵I-HDL and unlabeled LDL at the indicated molar ratio to ¹²⁵I-HDL (○). After further incubation for 1 h at 0°C, the cells were harvested and surface binding of labeled lipoprotein was determined as described in Methods. Results are expressed as percentages of values obtained in the absence of unlabeled lipoprotein (for ¹²⁵I-LDL, 94±6.1; for ¹²⁵I-HDL, 3.3±0.3). Each point represents the mean ±SEM of measurements performed in triplicate. At each molar ratio examined the reduction of ¹²⁵I-HDL binding by LDL was significantly greater (*P* < 0.005) than the reduction of ¹²⁵I-LDL binding by HDL. Specific activities: ¹²⁵I-LDL, 232 cpm/ng; ¹²⁵I-HDL, 220 cpm/ng. Molar conversion factors appear in the legend to Fig. 1.

DISCUSSION

The uptake and degradation of LDL by normal human fibroblasts have been previously characterized by Goldstein and Brown and their co-workers, who have presented conclusive evidence that these processes are important for the regulation of cell cholesterol synthesis and content (2, 7-10, 39). The present studies demonstrate that the binding, internalization, and degradation of LDL by fibroblasts are influenced by HDL. At very low molar ratios of HDL to LDL, HDL produced small increases in LDL uptake and degradation. At HDL:LDL molar ratios of 5:1 or greater, however, there was always a significant reduction in LDL uptake and degradation, the effect increasing progressively with increases in the HDL:LDL ratio. This was accompanied by a definite reduction of the net increase in cell cholesterol content induced by LDL. In contrast to HDL, human albumin and γ-globulin had no effect on LDL metabolism. Brown and Goldstein have also observed an inhibition of

LDL uptake by fibroblasts in the presence of high concentrations of HDL (40). These investigators, however, examined neither the underlying mechanism nor the consequences of the interaction with regard to the LDL-induced changes in cell cholesterol metabolism.

Consideration must be given to the possibility that the HDL:LDL interaction was an artifact due to contamination of the lipoprotein preparations with other lipoprotein classes. This is ruled out by the following quantitative considerations. Using ¹²⁵I-LDL at 4.9 μg protein/ml, the addition of an equal concentration of unlabeled LDL reduced both binding and degradation by 32%; the addition of HDL at 19.6 μg protein/ml (molar ratio, 20:1) reduced LDL binding by 29% and degradation by 31%, i.e., to about the same extent as did 4.9 μg LDL protein/ml. Thus, the HDL preparation would have had to contain about 25% LDL if LDL contamination were the basis for the effect; radioimmunoassay showed that our HDL preparation contained <0.5% immunoreactive B apoprotein.

The observed reduction of LDL internalization by HDL could have resulted from reduced surface binding of LDL, from inhibition of the internalization process, or both (2, 3, 7, 40). Evidence for the former mechanism

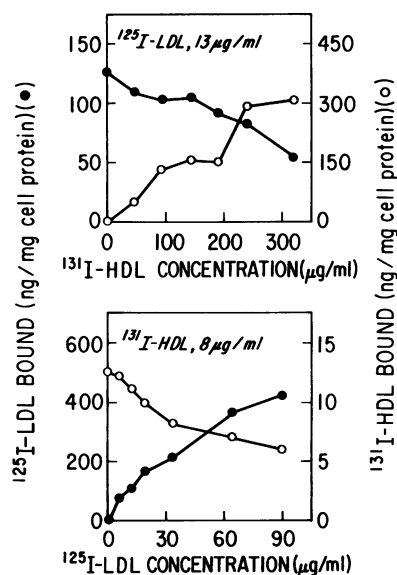


FIGURE 5 Reciprocal changes in the binding of ¹²⁵I-LDL and ¹³¹I-HDL to normal human fibroblasts associated with alterations in the ¹²⁵I-LDL:¹³¹I-HDL ratio in the culture medium. Cell monolayers were incubated for 18 h in medium containing 5% LDS. This was then replaced with similar medium containing either 13 μg/ml of ¹²⁵I-LDL and the indicated concentration of ¹³¹I-HDL (upper panel), or 8 μg/ml of ¹³¹I-HDL and the indicated concentration of ¹²⁵I-LDL (lower panel). After a 3-h incubation at 0°C, the cells were harvested and the binding of ¹²⁵I-LDL (●) and ¹³¹I-HDL (○) separately determined, using double-isotope counting, as described in Methods. Specific activities: ¹²⁵I-LDL, 260 cpm/ng; ¹³¹I-HDL, 188 cpm/ng.

is provided by the observation that HDL reduced LDL binding at 0°C at which temperature little or no internalization takes place and that similar effects were obtained when trypsin-releasable LDL was measured, which has been shown to represent primarily LDL bound to the cell surface (3, 4, 30). Furthermore, the reduction in LDL internalization and degradation was directly proportional to that in surface binding ($r = +0.97, P < 0.001$; Fig. 1). A primary effect on surface binding is also supported by the failure of HDL to inhibit the cellular uptake of sucrose, an index of fluid pinocytosis (32, 33), at concentrations which in other experiments produced a marked reduction in the rate of uptake of LDL. Because degradation of LDL apparently occurs only after entry of the lipoprotein into the cell (8, 41), the inhibition of LDL degradation by HDL can probably be accounted for primarily or exclusively by the reduction in the rate of internalization, and this in turn may be largely due to the reduction in surface binding.

The intimate mechanism by which HDL reduces the binding of LDL cannot be completely ascertained from the present experiments. The finding that preincubation of fibroblasts with HDL was as effective in reducing the binding of ^{125}I -LDL as the simultaneous addition of HDL and ^{125}I -LDL to the medium suggests that the HDL-LDL interaction was occurring at the cell membrane rather than in solution. We are not aware of any strong HDL-LDL interactions in solution but cannot rule out some contribution of that kind to the results observed. The demonstration that at a given HDL: ^{125}I -LDL ratio the interaction occurred equally over a wide range of ^{125}I -LDL concentrations suggests that HDL-LDL interaction occurs at both low affinity and high affinity LDL binding sites. Interaction at low affinity sites is confirmed by the finding that HDL reduced the binding of LDL to fibroblasts from a patient with HFH, cells that have been reported to lack high affinity receptors for LDL (2, 7, 9, 40). HDL-LDL interaction also occurred, and occurred to a comparable extent, in normal fibroblasts in which the number of high affinity LDL receptors had been reduced (2, 11) by preincubation with pronase or with sterols.

One possible mechanism for the interaction is that LDL and HDL compete for some common binding sites (although other sites may independently bind LDL or HDL exclusively). Bersot et al. (42) have presented evidence that a cholesterol-rich lipoprotein isolated from plasma of cholesterol-fed swine, HDL_c, competes effectively with human LDL for high affinity binding to human fibroblasts. HDL_c contains no apolipoprotein B but contains more arginine-rich peptide than does normal HDL. A competitive HDL-LDL interaction would be consistent with the reduction of ^{125}I -HDL uptake and degradation by

TABLE IV
Effects of HDL on the Changes Induced by LDL
in Cell Cholesterol Content

| Lipoprotein additions to 5% LDS medium* | Cell cholesterol content $\mu\text{g}/\text{mg protein}$ | P value† |
|--|---|----------|
| None | 27.9±0.9 | — |
| HDL only, 978 $\mu\text{g}/\text{ml}$ | 29.8±1.1 | NS |
| LDL only, 102 $\mu\text{g}/\text{ml}$ | 38.6±1.9 | 0.001 |
| LDL, 102 $\mu\text{g}/\text{ml}$ + HDL, 978 $\mu\text{g}/\text{ml}$ | 30.8±1.3 | NS |

Cell monolayers were incubated for 18 h in medium containing 5% LDS. The medium was then replaced with fresh medium containing 5% LDS and the indicated concentration of LDL and/or HDL, and the incubation was continued for 18 h. The cells were washed, harvested, and the cholesterol content was measured by gas chromatography as described in Methods. Results are expressed as the mean±SEM of values obtained from four or five replicate dishes.

* Lipoprotein concentrations are given in terms of protein content.

† Significance of the differences of the means between cholesterol content of cells exposed to LDS only and that of cells exposed to lipoproteins.

LDL, and with the reciprocal changes in ^{131}I -HDL and ^{125}I -LDL binding observed with alterations in the ^{131}I -HDL: ^{125}I -LDL ratio in the medium. These interactions could relate to specific apoproteins, such as the arginine-rich protein, present as integral components of HDL or to some subset of molecules isolated with HDL by the differential density methods used.

Studies in this laboratory have suggested that HDL binds mostly to sites other than the high affinity receptor for LDL. Thus the binding of HDL to normal fibroblasts was little affected by preincubation of the cells with either pronase or 7-ketocholesterol (43, 44) both of which produced a marked reduction in the number of high affinity sites for LDL, in agreement with the findings of Brown and Goldstein (11). Furthermore, neither the high affinity nor the low affinity binding of HDL was reduced in fibroblasts from a patient with HFH (1). Such observations, however, do not completely exclude the possibility that HDL and LDL may bind in part to common receptors. The experiments with ^{125}I -LDL and ^{131}I -HDL (Fig. 5) showed that the number of HDL molecules bound to the cell surface was more than 20 times the total number of LDL molecules displaced from high affinity sites. Thus, no more than 5% of the HDL molecules bound would have had to be bound to high affinity LDL sites (or bound in a manner that influences LDL binding to high affinity sites). Consequently, there would be no necessary inconsistency between an

apparently competitive inhibition by HDL of LDL binding to high affinity sites, on the one hand, and the observations that overall HDL binding is not reduced in HFH cells, which lack high affinity LDL binding sites, or in pronase-treated cells, in which the high affinity LDL binding sites have been largely destroyed. The difference expected (<5%) would be too small to detect. Other possible mechanisms for the HDL-LDL interaction include alteration of affinities for LDL due to site occupancy by HDL (negative cooperativity; reference 45) and steric hindrance during the binding of HDL and LDL to different but adjacent sites. In relation to the latter, the greater molecular size of LDL (37) may explain its greater effectiveness in reducing the binding of HDL compared to the effectiveness of HDL in reducing the binding of LDL (Fig. 4).

The cholesterol content of cultured cells is determined by the balance among the uptake of lipoprotein cholesterol, endogenous cholesterol synthesis, and cellular cholesterol efflux. Incubation of fibroblasts with LDL increases their cholesterol content as a result of internalization of the lipoprotein, even though cholesterol synthesis is inhibited (3, 5, 9, 10, 39). Although the surface binding of HDL is quantitatively similar to that of LDL, HDL is much more slowly internalized and degraded (1). Furthermore, the amount of cholesterol transported into the cell with each molecule of HDL is much less than that for LDL (35, 36). Accordingly, as reported by Brown et al. (39) and confirmed in this study, incubation of fibroblasts with HDL produces little or no increase in cell cholesterol content. Because the binding of HDL reduces the internalization of LDL, the total uptake of cholesterol in the presence of both lipoproteins would be expected to be less than that with LDL alone. The finding that HDL antagonized the net increase in cell cholesterol content is consistent with such an effect. Alternatively, HDL may have promoted the efflux of LDL cholesterol as proposed by Glomset (18). Both mechanisms may contribute.

The HDL:LDL molar ratio in the plasma of North American adults ranges from approximately 5:1 to 25:1 (37). Moreover, studies of peripheral lymph in animals have indicated that the HDL:LDL ratio in interstitial fluid is higher than that in plasma (46). Thus, the HDL:LDL molar ratios to which peripheral cells are exposed *in vivo* are likely to be in a range similar to those at which a significant reduction in LDL uptake was observed in the present study. An inhibition of LDL uptake by HDL has been reported in cultured swine smooth muscle cells (20) and has also been noted in recent studies with vascular endothelium (6, 47, 48). Thus, a high concentration of HDL in plasma could lead to a reduction in the peripheral uptake of LDL: (a) by limiting transfer across the endothelial cell

lining; and (b) by limiting uptake from the interstitial fluid.

Recent *in vivo* studies have demonstrated that the body pool of exchangeable cholesterol in hyperlipidemic human subjects is negatively correlated with the plasma HDL cholesterol concentration (17). There is also increasing epidemiologic evidence for an important inverse relationship between plasma HDL concentration and coronary heart disease risk (49–51). The HDL:LDL interaction provides one possible explanation for these relationships but additional studies are needed to determine whether such interactions occur to a significant extent *in vivo*.

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