Uptake and degradation of high density lipoprotein: comparison of fibroblasts from normal subjects and from homozygous familial hypercholesterolemic subjects

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Abstract Fibroblasts cultured from the skin of subjects with homozygous familial hyperlipoproteinemia (HFH) internalize and degrade low density lipoproteins at a much lower rate than do fibroblasts from normal subjects. Evidence has been presented that this reflects the absence from such mutant cells of specialized binding sites with high affinity for low density lipoproteins. The specificity of this membrane defect in familial hypercholesterolemia is further supported by the present studies comparing the metabolism of low density lipoproteins (LDL) and high density lipoproteins (HDL) in normal fibroblasts and in fibroblasts from HFH patients. The surface binding (trypsin-releasable ^{125}I) of ^{125}I-labeled LDL by HFH cells was approximately 30% of that by normal cells at a concentration of 5 μg LDL protein per ml. At the same concentration the internalization (cell-associated ^{125}I after trypsinization) and degradation (trichloroacetic acid-soluble non-iodide ^{125}I) of ^{125}I-labeled LDL were less than 10% of the values obtained with normal cells. In contrast, the binding of ^{125}I-labeled HDL to HFH cells was actually somewhat greater than that to normal cells. Despite this, the internalization and degradation of ^{125}I-labeled HDL by HFH cells averaged only 70% of that by normal cells. [^{3}H]- or [^{14}C]Sucrose uptake, a measure of fluid uptake by pinocytosis, was similar in normal and HFH fibroblasts. These findings are consistent with the proposal that fibroblasts from subjects with HFH lack high-affinity receptors for LDL. These receptors do not play a significant role in HDL binding and uptake. Instead, as previously proposed, HDL appears to bind randomly on the cell surface and its internalization is not facilitated by the specific mechanism that internalizes LDL. The small but significant abnormalities in HDL binding and internalization, however, suggest that there may be additional primary or secondary abnormalities of membrane structure and function in HFH cells. Finally, the observed overall rate of uptake of LDL (that internalized plus that degraded) by HFH fibroblasts was considerably greater than that expected from fluid endocytosis alone. This implies that adsorptive endocytosis, associated with binding to low-affinity sites on the cell surface, may play a significant role in LDL degradation by HFH cells, even though it does not regulate endogenous cholesterol synthesis in these cells.

Supplementary key words low density lipoprotein · high-affinity binding sites · fluid endocytosis · adsorptive endocytosis

When fibroblasts cultured from normal human subjects are transferred from their usual medium, containing whole serum, and are incubated for some time in a medium containing lipoprotein-deficient serum, there is a marked increase in the rate of cholesterol synthesis (1–3). If low density lipoprotein (LDL) is then added to the medium, the rate of cholesterol synthesis declines because of suppression of the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase) (1–3). Brown, Goldstein and co-workers (4, 5) have shown that this is mediated by free cholesterol released from LDL cholesteryl esters in the cells during uptake and degradation of the LDL. In contrast, fibroblasts from subjects with homozygous familial hypercholesterolemia (HFH) do not show suppression of HMG CoA reductase activity and sterol synthesis even in the presence of very high concentrations of LDL (1, 2, 6). This is related to a gross defect in the uptake and degradation of LDL (4, 6). It has been postulated that this is due to the

Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein; HFH, homozygous familial hypercholesterolemia; FCS, fetal calf serum; LDS, lipoprotein-deficient serum; DME, Dulbecco's modification of Eagle's minimal essential medium; ^{125}I-LDL(HDL), ^{125}I-labeled LDL (HDL).

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absence from the HFH cells of a specific receptor for LDL normally present in the cell membrane (4). Consistent with this proposal is the demonstration that the high-affinity binding of LDL to the surface of HFH fibroblasts is less than that to normal fibroblasts, although the magnitude of the difference appears to vary according to the method used for releasing LDL from the cell membrane (6, 7). Moreover, the LDL binding sites on normal cells differ qualitatively from those on HFH cells. Goldstein and Brown (4) showed that treatment with pronase destroys high-affinity LDL binding sites on normal cells but does not reduce LDL binding to HFH cells, a finding confirmed in this laboratory for the HFH line (P.A.) used in the present studies (8). In normal cells the high-affinity receptor number is regulated by LDL itself or by sterols, as shown by Brown and Goldstein (9); no such regulation was found for binding of LDL to HFH cell line P.A. (8). Finally, Brown and Goldstein (10) and Goldstein, Brown, and Stone (11) have described a new mutation, clinically not distinguishable from HFH, in which the fibroblasts have a partial defect in binding and a total defect in internalization. This powerful genetic evidence added to that briefly summarized above leaves little doubt that in most HFH patients, including P.A., the primary defect is in the complete or nearly complete deficiency of a high-affinity receptor for LDL.

Previous studies in this laboratory showed that high density lipoprotein (HDL) also binds to normal human fibroblasts, and that the overall density of binding is comparable to that of LDL at equimolar concentrations (12, 13). In contrast to the LDL, however, HDL was only slowly internalized and degraded. Qualitative differences in the binding of the two lipoproteins suggested that HDL binds mostly to sites other than those to which LDL binds with high affinity (8, 14). In the present report we describe comparative studies on the binding, internalization, and degradation of HDL by fibroblasts from normal and HFH subjects. A preliminary report of part of this work appeared elsewhere (12).

METHODS

Materials

Sodium [^{125}I]iodide (carrier-free in 0.05 N NaOH) was obtained from Schwarz-Mann, Orangeburg, NY, D-[U-^{14}C]sucrose (440 mCi/nmol) from New England Nuclear Corporation, Boston, MA, and 6,6′(*n*)-[^{3}H]sucrose (4 Ci/mmol) from Amersham Searle Corp., Arlington Heights, IL. Fetal calf serum was purchased from Irvine Scientific Sales Company. Dulbecco's modification of Eagle's minimal essential medium (DME) (15) and Dulbecco's phosphate buffered saline (PBS) (16) were obtained from Gibco.

Cell cultures

Skin fibroblasts were grown in monolayer from a preputial biopsy of a normal infant (B.B.) and from skin biopsies of a 19-year-old female (P.A.) and a 12-year-old (J.P.) with classical HFH. The clinical features and family histories of the subjects with HFH have been documented (P.A.: subject P_4 in ref. 17 and subject 2 in ref. 18; J.P.: described in ref. 2.). Studies of the metabolism of LDL by each of these cell lines have already been reported (4, 6, 19). Cultures were maintained in a humidified incubator (95% air, 5% CO_2) at 37°C in DME containing 23 mM $NaHCO_3$, 0.3 mg/ml glutamine, and 10% (v/v) fetal calf serum (FCS) (final protein concentration, 5 mg/ml). Cells were studied between the 9th and 20th passages. Polystyrene tissue culture dishes (60 × 15 mm; Corning) were seeded with 1–2 × 10^5 cells and used 4–6 days later, at which time the cultures were 50–95% confluent. Growth curves have previously shown that the normal cell line reaches a plateau at 1–2 × 10^6 cells per dish in 6–8 days (6). The mutant cells are fewer in number at confluency (4–8 × 10^5 cells/dish). At the time of study, cell protein per dish was 250–550 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 (low density lipoprotein) and 1.090–1.21 g/ml (high density lipoprotein) were isolated from pooled fasting human plasma by preparative ultracentrifugation (20) following procedures previously described to avoid cross-contamination (13, 14).

Isolated fractions were dialyzed against buffer containing 20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, and 0.3 mM EDTA. They were then sterilized by passage through a Millipore filter (0.22 or 0.45 μm) and stored at 4°C. Protein content was determined by the method of Lowry et al. (21). Lipoprotein fractions were shown to be free of cross-contamination with other lipoprotein classes by immunodiffusion (22) against specific rabbit antisera to human LDL and HDL. The sensitivity of the method, tested with serial dilutions of LDL, would have allowed the detection of as little as 1% LDL contamination of HDL fraction. The B apoprotein content of the HDL fraction, measured for us by Dr. John J. Albers by radioimmunoassay, was less than 0.5% of the

total protein in the fraction. Lipoprotein-deficient fetal calf serum (LDS) was prepared and used as described previously (3).

^{125}I-Labeled HDL and LDL were prepared by a modification of the iodine monochloride method of McFarlane (23) as previously described (3). Specific activities were 198–561 cpm/ng protein for ^{125}I-HDL. Less than 3% of the total ^{125}I in the final preparations was TCA-soluble and less than 2% was extractable into chloroform-methanol 2:1 (v/v). More than 93% of 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 on Sephadex G-100 (24) showed that 53% of radioactivity was in apoprotein A-I, 42% was in apoprotein A-II, and 5% was in C peptides. No ^{125}I-labeled lipoprotein was used more than 4 weeks after its preparation.

The integrity of ^{125}I-HDL was assessed by diluting it progressively (up to 20-fold) with unlabeled HDL, while holding the total HDL concentration constant at 23 μg/ml, and examining the effects on binding, internalization, and degradation of the radioactive HDL by fibroblasts as described below. These parameters all decreased in direct proportion to the extent of dilution, suggesting that the cells did not distinguish between labeled and unlabeled lipoprotein. Similar results have been previously reported for ^{125}I-LDL prepared as described here (6). Recentrifugation of ^{125}I-HDL and ^{125}I-LDL at densities of 1.21 and 1.063 g/ml, respectively, prior to their use was found to have no appreciable effect on the binding, internalization, and degradation of either lipoprotein.

Lipoprotein uptake and degradation

Eighteen hours before an experiment, the medium was removed from each dish and replaced with 3 ml of fresh medium containing 5% (v/v) LDS. Immediately before the experiment this was replaced by 2 ml of fresh medium of the same composition. In studies carried out at 0°C, the dishes were placed on crushed ice for 15 min before the start of the experiment and then held on ice in a 4°C cold room. Other incubations were performed at 37°C in a humidified incubator (95% air, 5% CO_2).

At the end of the incubation with ^{125}I-labeled lipoprotein, the medium was removed and an aliquot assayed for total ^{125}I. Trichloroacetic acid was added to the remaining medium to a final concentration of 10% and the mixture was heated to 100°C for 5 min. After centrifugation (3000 *g* for 10 min) an aliquot of the supernate was treated to remove free iodide by oxidation with H_2O_2 and extraction of I_2 into chloroform (25). All degradation data refer to non-iodide, TCA-soluble radioactivity. Net degradation was calculated as the difference between values obtained from identical incubations in the presence and absence of cells. It was shown that there was no intracellular accumulation of degradation products (TCA-soluble, non-iodide ^{125}I in the cells accounted for less than 20% of total cell radioactivity after an 18-hr incubation and less than 5% of the TCA-soluble, non-iodide ^{125}I accumulated in the medium).

After removal of the medium, the cells were washed six times with 2 ml of PBS at 0°C. Two ml of 0.05% trypsin in Versene buffer was added to each plate and the plates were incubated at 37°C for an additional 3–4 min. The cells were collected and the plates were scraped with washings of two 1-ml aliquots of DME containing 10% FCS (to arrest the action of the trypsin). The cells were sedimented by centrifugation (3000 *g* for 10 min) at 4°C, and an aliquot of the supernate was assayed for ^{125}I radioactivity. The ^{125}I released by trypsin was considered to represent lipoprotein bound to the cell surface (6, 13, 25, 26). Incubations without cells showed that trypsinization did not result in an important release of ^{125}I-HDL or ^{125}I-LDL adsorbed to the dish (4–8% of that released from identical dishes containing cells). The amount of radioactivity in the final PBS wash was shown to be less than 4% of that subsequently released by trypsin.

The cells were washed by suspension in 4 ml of PBS and centrifugation at 3000 *g* for 20 min. The pellet was dissolved in 0.2 ml of 1 N KOH (20°C, 24 hr) and assayed for ^{125}I. The cell digest was then diluted with water to 1 ml; aliquots were removed for protein assay (21), measurement of total lipid-soluble radioactivity (chloroform-methanol 2:1 v/v) and TCA-soluble radioactivity (final concentration, 10%). The TCA-precipitable, nonlipid radioactivity remaining associated with the cells after trypsinization was considered to represent internalized lipoprotein (6, 25, 26).

Lipoprotein concentrations and data on binding, internalization, and degradation are expressed in terms of lipoprotein protein.

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

The uptake of radiolabeled sucrose as a measure of fluid pinocytosis (27, 28) was measured as described previously (14). Briefly, cells were incubated in medium containing D-[^{14}C(U)]sucrose at a concentration of 1.1 μmol/l (0.5 μCi/ml). Medium was removed and an aliquot was added to 15 ml of scintillation solution (10.5 ml of toluene, 4.5 ml of Triton, 60 mg of 2,5-diphenyloxazole, 0.8 mg of

[1,4-bis-2-(5-phenyloxazolyl)-benzene]) for determination of ^{14}C radioactivity. At the end of the incubation the cells were washed 10 times with 3 ml of PBS, harvested with a rubber policeman, and dissolved by allowing to stand overnight on 0.2 ml of 1 N NaOH. Aliquots were taken for protein determination (21) and for liquid scintillation counting as previously described (14).

RESULTS

The time course for binding, internalization, and degradation of HDL by HFH fibroblasts at 37°C was similar to that previously reported for normal cells (14). Surface binding was rapid and showed little increase beyond 1–2 hr and internalization reached a plateau by 6–12 hr, but degradation proceeded linearly for at least 24 hr (after an initial lag period of 30 min). The temperature dependency of HDL binding, internalization, and degradation by HFH fibroblasts was also similar to that already described for normal cells (14). At 0°C, internalization and degradation were almost completely abolished but surface binding was only slightly less than that seen at 37°C (data not shown).

The results of a representative experiment comparing the binding of LDL and HDL to normal and to HFH cells at 0°C as a function of lipoprotein concentration are shown in **Fig. 1.** In accordance with the reports of Brown, Goldstein and coworkers (4, 7) and results reported from this laboratory (6), the binding of LDL by fibroblasts from subjects with HFH was less than that by normal cells. In contrast, the binding of HDL by the HFH cells over a similar range of molar concentrations[3] was somewhat greater than that by normal cells.

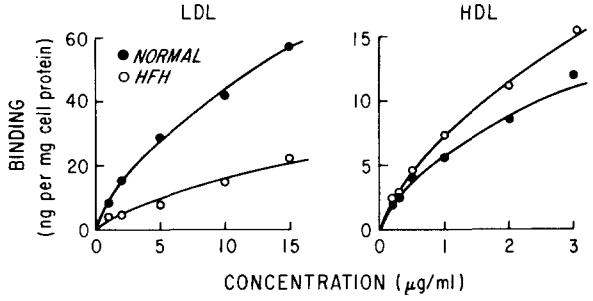

Fig. 1. Surface binding of HDL and LDL at similar molar concentrations by normal fibroblasts (●) and by fibroblasts from a patient with homozygous familial hypercholesterolemia (○). Fibroblast monolayers were incubated for 18 hr in medium containing 5% LDS (v/v). This was then replaced with fresh medium containing 5% LDS and the indicated concentration of ^{125}I-LDL (left panel) or ^{125}I-HDL (right panel). After further incubation for 2 hr at 0°C, the cells were harvested for measurement of surface-bound lipoprotein as described under Methods. Each point represents the mean value from two or three dishes. Specific activities: ^{125}I-LDL, 219 cpm/ng; ^{125}I-HDL, 198 cpm/ng. Cell protein per dish: normal cells, 311 ± 32 μg; HFH cells, 265 ± 37 μg (mean ± SD).

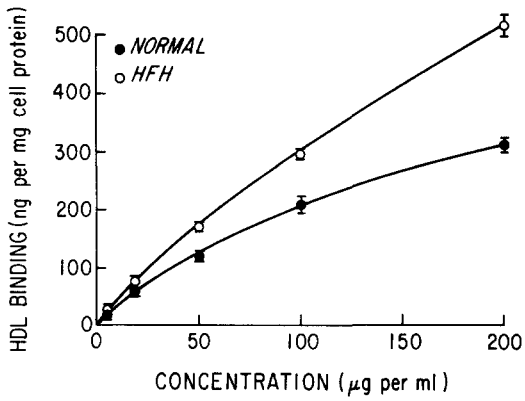

Fig. 2. Surface binding of HDL by normal fibroblasts (●) and by fibroblasts from a patient with homozygous familial hypercholesterolemia (○) over a wide range of HDL concentrations. Fibroblast monolayers were incubated for 18 hr in medium containing 5% LDS. This was then replaced with fresh medium containing 5% LDS and the indicated concentration of ^{125}I-HDL (198 cpm/ng). After further incubation for 2 hr at 0°C, the cells were harvested for determination of HDL binding as described under Methods. Each point represents the mean ± SEM from incubations performed in triplicate. Cell protein per dish: normal cells, 351 ± 28 μg; HFH cells, 308 ± 21 μg (mean ± SD).

The absolute difference between HDL binding to normal and to mutant cells increased with increasing HDL concentration in the medium. This is illustrated in **Fig. 2,** which compares the 0°C binding of HDL by normal and HFH fibroblasts over a wider range of concentrations, from 5 to 200 μg HDL protein/ml. Similar results were obtained with the other mutant cell line studied and in experiments performed at 37°C (data not shown).

The curvilinear relationship between HDL binding and HDL concentration shown in Fig. 2 suggests that the surface binding to normal fibroblasts occurs with greater affinity at low than at high concentrations. Scatchard plots (34) of the data shown in Fig. 2, together with additional data collected in the same experiment at HDL concentrations between 0.2 and 5 μg/ml, are presented in **Fig. 3.** Curve analysis by the method of least squares indicated that the difference between the two curves was consistent with an approximately two-fold increase (per mg of cell protein) in the number of apparent low-affinity binding sites for HDL on the surface of the HFH cells, but with

[3] Molar lipoprotein concentrations were calculated assuming a molecular weight of 550,000 for LDL protein (30, 31) and a molecular weight of 110,000 for HDL protein (32, 33). Thus a 5:1 molar ratio of HDL to LDL corresponds to a 1:1 protein concentration ratio.

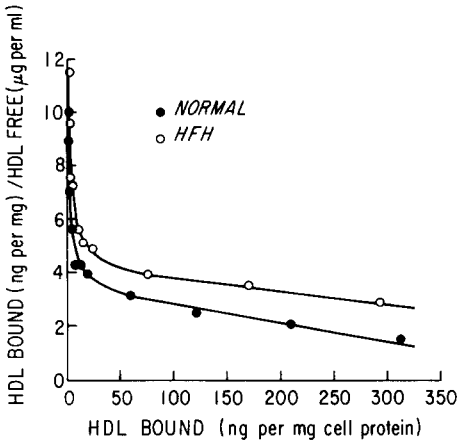

Fig. 3. Scatchard plots of HDL binding to normal fibroblasts (●) and to fibroblasts from a patient with homozygous familial hypercholesterolemia (○) at HDL concentrations ranging from 0.2 to 200 μg/ml. Each point represents the mean of triplicate determinations. Experimental details are as given in the legend to Fig. 2. Cell protein per dish: normal cells, 321 ± 38 μg; HFH cells, 293 ± 27 μg (mean ± SD).

no marked difference between the cell lines in the number of high-affinity sites or in the binding affinities of the two classes of sites.

We have previously shown that the rates of internalization and degradation of HDL by normal fibroblasts at 37°C are very low relative to those of LDL (14). The internalization and degradation of HDL by normal and HFH cells were compared using incubations of 18 hr duration (Figs. 4 and 5). The internalization of HDL by normal and HFH cells as a function of concentration are directly compared with those of LDL over a similar range of molar concentrations in **Fig. 4.** In confirmation of earlier

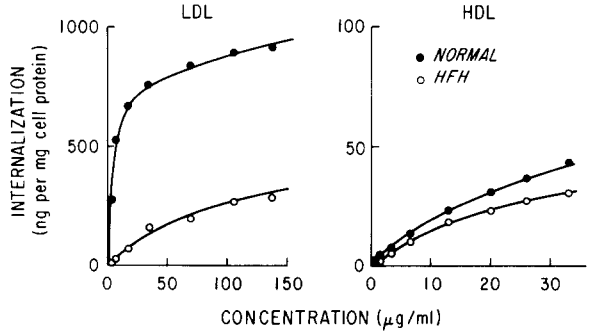

Fig. 4. Internalization of HDL and LDL at similar molar concentrations by normal fibroblasts (●) and by fibroblasts from a patient with homozygous familial hypercholesterolemia (○). Fibroblast monolayers were incubated for 18 hr in medium containing 5% LDS. This was then replaced with fresh medium containing 5% LDS and the indicated concentration of ^{125}I-LDL (left panel) or ^{125}I-HDL (right panel). After further incubation for 18 hr at 37°C the cells were harvested for measurement of lipoprotein internalization as described under Methods. Specific activities: ^{125}I-LDL, 333 cpm/ng; ^{125}I-HDL, 561 cpm/ng. Cell protein per dish; normal cells, 497 ± 23 μg; HFH cells, 299 ± 20 μg (mean ± SD).

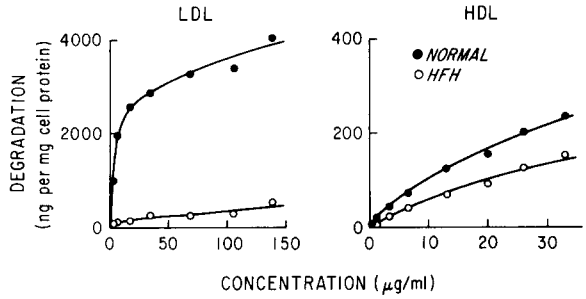

Fig. 5. Degradation of HDL and LDL at similar molar concentrations by normal fibroblasts (●) and by fibroblasts from a patient with homozygous familial hypercholesterolemia (○). Results were obtained from the same experiment as that illustrated in Fig. 4.

studies (6, 7), the internalization of LDL by HFH cells was found to be substantially less than that by normal cells, particularly at low LDL concentrations (below 20 μg/ml). In contrast, the internalization of HDL by HFH cells was only slightly less than that by normal cells. Similar results were consistently observed with both HFH cell lines. Studies of LDL and HDL degradation (**Fig. 5**) gave results that paralleled almost exactly the results for internalization.

Thus, both internalization and degradation of HDL by fibroblasts from subjects with HFH were somewhat less than in normal fibroblasts, even though the surface binding of HDL by the HFH cells was greater. The possibility that this may have reflected a generalized reduction of endocytosis in the mutant cells was examined by measuring the uptake of [^{14}C]sucrose. The results of one representative experiment are shown in **Fig. 6.** It can be seen that the uptake of sucrose, and therefore also of culture medium (27, 28), by the two cell lines was equal in incubations of 4–27 hr duration. In a series of 18-hour incubations, the clearance of medium water calculated from [^{14}C]sucrose uptake measurements was 3.4 μl ± 0.3 (SD; n = 14) for normal cells and 3.5 ± 0.5 for the mutant cells. The overall range of sucrose clearance in five different experiments with both normal and HFH fibroblasts was from 1.5 to 3.8 μl/ml per 18 hr but the range in any given experiment was much smaller. Steinman, Brodie, and Cohn (28) have shown that fluid endocytosis increases as fibroblast monolayers reach confluency. To determine the extent of "leakage" of sucrose, normal fibroblasts were incubated with [^{3}H]sucrose for 24 hr, washed, and then incubated for an additional 18 hr in sucrose-free medium. Less than 15% of the cell-associated labeled sucrose was released to the medium.

Goldstein and Brown (4) have previously estimated fluid endocytosis using ^{125}I-labeled albumin as a marker. If albumin is cleared exclusively by fluid

endocytosis, their data indicate clearances many times greater than the values obtained using sucrose as a marker (14). In an attempt to clarify the difference in results, we directly compared uptake of [^{3}H]sucrose and ^{125}I-labeled albumin (**Table I**). The albumin was labeled by the nonoxidative method of Wood, Wu, and Gerhart (29) and iodination was terminated when the specific activity of the albumin reached 22 cpm/ng. As shown in Table I, the apparent clearance of labeled albumin was 4–6 times that of sucrose; LDL (25 μg/ml) did not affect the clearance rate.

Knowing the [^{14}C]sucrose clearance, it is possible to calculate the ^{125}I-LDL uptake attributable to fluid endocytosis. The additional uptake due to adsorptive endocytosis can be calculated from the rate of sucrose clearance and measurement of LDL bound if we make certain assumptions about the mean diameter of the endocytotic vesicles (28). We have previously reported that observed total LDL uptake by normal fibroblasts (the amount internalized plus the amount degraded during an incubation) is far in excess of the uptake attributable to both fluid and adsorptive endocytosis calculated in this way (14), implying a specialized mechanism for LDL uptake. In contrast, observed HDL uptake was very close to the uptake calculated on the basis of the same assumptions. In **Table 2** are presented the results of an experiment in which LDL uptake by normal and by HFH cells was measured in quadruplicate and the results were calculated as described above. Observed LDL uptake by normal cells was orders of magnitude greater than that expected from fluid endocytosis alone; it was many-fold greater than the total calculated endocytotic uptake (by fluid plus adsorptive endocytosis). In contrast, the observed LDL uptake in HFH cells was in rather good agreement with the total calculated uptake by fluid plus adsorptive endocytosis. The observed LDL uptake by HFH cells was much too great to have been due to fluid endocytosis alone.

TABLE 1. Comparison of estimates of endocytosis using sucrose or using albumin as marker[a]

Addition to Lipoprotein-Deficient Medium	Calculated Volume of Medium Cleared	
	Normal fibroblasts	HFH fibroblasts
	µl/mg cell protein/24 hr	
A. [^{3}H]Sucrose	2.5	1.7
^{125}I-Labeled human albumin	10.3	11.3
B. [^{3}H]Sucrose + 25 µg/ml LDL-protein	2.6	1.8
^{125}I-Labeled human albumin + 25 µg/ml LDL-protein	10.6	9.1

[a] Cells were incubated for 24 hr at 37°C in the presence of either 2 µc/ml of 6,6′-[^{3}H]sucrose (4 Ci/mmol) or 10.2 µg/ml of ^{125}I-labeled human albumin (22 cpm/ng). Albumin uptake was measured as the sum of albumin degraded during the 24-hr incubation (medium radioactivity soluble in 10% trichloroacetic acid) and that associated with the washed cells. Values represent mean values from triplicate or quadruplicate dishes.

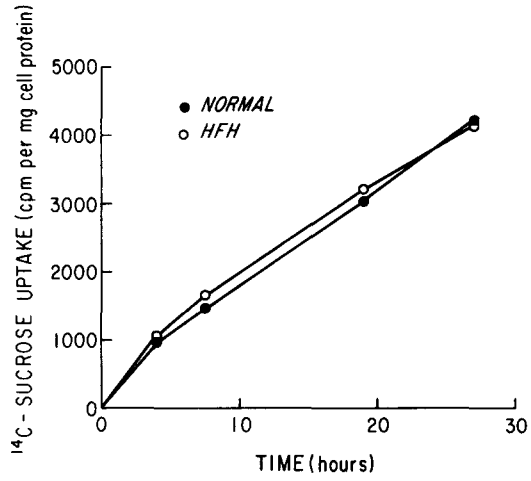

Fig. 6. Uptake of [^{14}C]sucrose by normal fibroblasts (●) and by fibroblasts from a patient with homozygous familial hypercholesterolemia (○) as a function of time of incubation. Cell monolayers were incubated for 18 hr in medium containing 5% LDS. This was then replaced with fresh medium containing 5% LDS and 0.5 µCi/ml of [^{14}C(U)]D-sucrose (440 mCi/mmol). After further incubation at 37°C for the indicated periods of time, cells were harvested for measurement of ^{14}C radioactivity as described under Methods. Each point represents the mean of duplicate determinations. Cell protein: normal cells, 325 ± 58 µg; HFH cells, 288 ± 23 µg (mean ± SD).

DISCUSSION

In the present experiments the surface binding of LDL by fibroblasts from two subjects with HFH was clearly less than that by normal fibroblasts, the difference being most marked at LDL concentrations below 20 µg/ml. In contrast, the HFH cells showed no reduction in the binding of HDL. This result is consistent with the proposal of Goldstein and Brown that the binding defect in HFH cells is specific for LDL (4, 19).

The number of apparent binding sites for HDL was actually somewhat greater on the HFH cells than on the normal cells. The explanation for this is uncertain. One possibility is that it is a consequence of the relatively greater surface area of the HFH cells. As noted above, confluent monolayers of HFH fibroblasts contained only half as many cells as those of normal fibroblasts. Previous studies in this laboratory have shown that the protein content per cell of normal and HFH fibroblasts is essentially the same

TABLE 2. Observed LDL uptake versus calculated uptake by fluid and adsorptive endocytosis: comparison of normal cells and HFH cells[a]

	Medium LDL Conc.	Observed LDL Bound	Calculated Uptake			Observed Uptake (internalized and degraded)
			By Fluid Endocytosis[b]	By Adsorptive Endocytosis[c]	Sum	
	μg/ml	*ng/mg*	*ng/mg cell protein/18 hr*			*ng/mg/18 hr*
Normal cells	0.5	14.9 ± 1.2	1.8	70	72	308 ± 37
	2.5	61.4 ± 5.1	9	287	296	1349 ± 114
	12.5	218 ± 20.8	45	1019	1064	3125 ± 301
HFH cells	0.5	7.9 ± 0.40	1.8	24	26	16.8 ± 1.0
	2.5	36 ± 3.7	9	110	119	106 ± 6.1
	12.5	193 ± 8.7	45	593	638	530 ± 25

[a] Cells were incubated 18 hr at 37°C, quadruplicate dishes at each concentration of ^{125}I-LDL. Average protein content per dish: normal cells, 443 μg; HFH cells, 304 μg.

[b] A value of 3.6 μl/mg cell protein/18 hr was used for these calculations. Observed values were: normal cells, 3.40 ± 0.30 (SD; n = 14); HFH cells, 3.53 ± 0.48 (SD; n = 8).

[c] Density of lipoproteins binding to cell surface (ng/μm^2) × total area of cell surface internalized in 18 hr due to pinocytosis (μm^2/mg cell protein/18 hr). The formula proposed by Steinman, Brodie, and Cohen (28) for estimating true surface area ($3\pi D^2$) was used. Mean cell diameter of 25 μm was assumed for normal cells; 31 μm for HFH cells. The larger value for HFH cells is based on the observed lesser number of cells per dish at confluency (6). Mean surface area and volume of pinocytotic vesicles were assumed to be the same as in the mouse fibroblast L cell line studies by Steinman et al. (28) (area: 0.162 μm^2; volume, 0.00847 μm^3).

(6). Thus, it follows that the HFH cells presented twice as much surface area per mg of protein as normal cells. The magnitude of this difference in surface area between the normal and HFH fibroblasts is similar to that in the number of HDL binding sites per mg of cell protein, as determined by Scatchard analysis.

The validity of the Scatchard analysis depends on the validity of the assumptions underlying such an analysis. These include the assumptions that there are no interactions among the binding sites and that each ligand molecule interacts reversibly with only one binding site. Because LDL and HDL probably contain multiple subunits distributed over a large surface, it is quite possible that each macromolecular complex interacts at several points with binding sites on the cell membrane. Furthermore, the curvature of the Scatchard plot might reflect not multiple classes of binding sites but rather negative cooperativity among them (35). In either case the conclusions drawn regarding number of binding sites would be invalidated.

Other studies in this laboratory (13, 14) have shown that the binding sites on human fibroblasts for HDL, in contrast to those for LDL (4), are relatively resistant to mild proteolytic digestion. This suggests either that HDL binds mostly to lipid or carbohydrate sites rather than to protein moieties or that any protein involved is not readily accessible to the protease. This conclusion is consistent with the finding of Jackson et al. (36) that the binding of one of the major apoproteins of human HDL, apoprotein A-II, to the surface of human erythrocytes was abolished following succinylation of the molecule, which would be expected to destroy its lipid-binding properties (37), but other interpretations are possible. The surface membrane of fibroblasts from subjects with HFH has recently been shown to have a greater content and exposure of certain glycosphingolipids (and also of glycoproteins) than that of normal fibroblasts (38). Thus, the greater binding of HDL to mutant than to normal cells in the present study may also have been related to such differences in the lipid or glycoprotein composition of their membranes.[4]

Despite the greater binding of HDL to the mutant cells, HDL internalization and degradation were somewhat less than in normal cells. These differences were much smaller, however, than those in LDL internalization and degradation. Thus, at a concentration of 0.1 nmol/ml (9 μg HDL protein/ml; 56 μg LDL protein/ml) the difference between normal and mutant cells in the total uptake of lipoprotein (internalized plus degraded) was only 0.5 nmol/g cell protein per 18 hr for HDL compared with 6.5 nmol/g cell protein per 18 hr for LDL (Figs. 4 and 5), corresponding to reductions of 30% and 90%, respectively. Nevertheless, the small reductions in HDL internalization and degradation were consistently observed with both mutant cell lines studied. The possibility must be considered that these may

[4] Fishman et al. (43), however, report finding no consistent differences in lipid composition of normal and HFH fibroblasts.

have reflected contamination of the HDL preparations with lipoproteins of lower density. However, this would appear to be unlikely, since the HDL preparations were checked by immunodiffusion against specific rabbit antiserum to human LDL and shown to contain less than 0.5% apoprotein B by radioimmunoassay. The density range of HDL employed (1.09–1.21 g/ml) would be expected to avoid contamination by sinking pre-β lipoproteins (density, 1.05–1.08 g/ml) (39).

We have previously shown (13) that the total rate of HDL uptake by normal fibroblasts is adequately accounted for by the sum of fluid endocytosis ("bulk" endocytosis) and adsorptive endocytosis (invagination of plasma membrane with its bound lipoprotein) if one assumes that binding and pinocytosis occur randomly over the whole cell surface. In contrast, the uptake of LDL greatly exceeded that attributable to fluid plus adsorptive endocytosis and therefore required the postulation of selective and favored internalization of LDL. Presumably this occurs at the specialized "coated pit" areas of the cell membrane identified by Anderson, Goldstein, and Brown (40). In the case of HFH fibroblasts, however, the uptake of LDL was adequately accounted for by the sum of fluid and adsorptive endocytosis. In other words, the binding and uptake of LDL by HFH fibroblasts was qualitatively similar to that of HDL by normal fibroblasts. Thus, in HFH cells there was no need to postulate non-random binding nor a specialized mechanism for internalization.

The validity of these calculations depends on the accuracy of the estimate of fluid endocytosis and of surface binding. The present results using sucrose as a marker extend and confirm our previously reported results (13); the values are in good agreement with those found by Steinman et al. (28) for several mouse fibroblast lines using horseradish peroxidase as the marker. Valid measurements of fluid endocytosis can only be made if the marker does not bind at all to the cell surface. The higher values found in the present studies using ^{125}I-labeled albumin as a marker suggest that it does bind to some extent. Moore, Williams, and Lloyd (41) have shown that albumin uptake can be markedly increased if the albumin is deliberately damaged even though it remains soluble and of unchanged molecular size.

Surface binding to monolayers of fibroblasts may be either overestimated or underestimated. It will be overestimated to the extent that the ligand binds to intercellular matrix or to surfaces of the plastic dish. Conversely, binding may be underestimated if the procedure for washing the cells removes ligand that is loosely bound to the cell surface. If the objective is the study of high-affinity binding, then the washing procedure should be vigorous to remove all ligand bound loosely. On the other hand, if the goal is to evaluate the magnitude of adsorptive endocytosis, even ligand bound loosely may be relevant. In the case of LDL, Anderson et al. (40) have demonstrated a category of binding that is unique, namely, the binding with high-affinity to the specialized "coated pit" areas which is associated with what we have called focal endocytosis. Binding to other areas on the cell surface, even though the binding is of low affinity, will lead to internalization above and beyond that accounted for by fluid endocytosis. Measurement of the difference between the maximum internalization due to fluid endocytosis, on the one hand, and observed internalization, on the other hand, provides a measure of such adsorptive endocytosis no matter what the affinity of the binding may be. In the present studies there happened to be reasonably good agreement between the observed internalization above and beyond that due to fluid endocytosis and the calculated increment expected on the basis of binding measured by the washing procedure used. Under other circumstances, the washing procedure might remove loosely bound LDL, leading to an underestimate of internalization associated with adsorptive endocytosis. It should be stressed that our estimates of adsorptive endocytosis do not depend on a direct measurement of the LDL adsorbed to the cell surface; uptake greater than that corresponding to fluid endocytosis (based on sucrose clearance data) must be attributed to one or another form of adsorptive endocytosis.

While the observed uptake of LDL by HFH cells was very low compared to that by normal cells, it was nevertheless much greater than that attributable to fluid endocytosis alone. Thus the LDL bound to the low affinity binding sites on these cells does appear to be internalized and degraded. As pointed out previously, if the concentration of LDL in the medium is made sufficiently great, the degradation rate even in HFH cells can be quite high (6). Nevertheless, endogenous cholesterol synthesis is not suppressed even though it would be in a normal cell degrading LDL at the same rate. Either the metabolism of LDL taken up from low affinity sites is somehow different; or the high-affinity LDL binding site plays some additional role in regulation of cell cholesterol metabolism; or HFH cells simply lose cholesterol more rapidly than normal cells, as proposed by Fogelman et al. (42).

Adsorptive LDL uptake from low affinity binding

sites, assuming it occurs also in arterial tissue, might be a factor in the pathogenesis of atherosclerosis in HFH. Such uptake does not lead to inhibition of endogenous sterol synthesis and thus appears to be qualitatively or, at least, quantitatively different from uptake at high affinity sites. In vivo, and possibly in other cell types, it could thus represent uncompensated delivery of sterol to the cell. Moreover, uptake of LDL may have additional metabolic consequences (delivery of other lipid components such as sphingolipids; effects on cell growth) relevant to atherogenesis.

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