Receptor Binding of Cholesterol-Induced High-Density Lipoproteins Containing Predominantly Apoprotein E to Cultured Fibroblasts with Mutations at the Low-Density Lipoprotein Receptor Locus†

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ABSTRACT: Previous equilibrium and kinetic studies have shown that cholesterol-induced high-density lipoproteins (HDL_c), which contain only the arginine-rich (E) apoprotein (apo-E), have an affinity for normal human fibroblasts that was 20 times that of human low-density lipoprotein (LDL). Also, 4 times as many LDL particles as HDL_c particles were required to saturate the normal human fibroblast surface receptors. The most feasible explanation was that each HDL_c particle bound to approximately four receptors. Here we considered two other possibilities: (a) that HDL_c bind to one receptor, suppressing three other receptors and (b) that HDL_c and LDL bind to different but adjacent receptors, such that binding of either blocks the binding of the other. The possibility of suppressed receptors proved unlikely because cells that had HDL_c bound and then removed could still bind the full complement of LDL. The possibility of blocked adjacent receptors was examined by using cell strains with genetic mutations in the LDL receptor locus. If the receptors for LDL and apo-E HDL_c were different, a mutation that altered the binding of one would not necessarily alter the binding of the other. The binding of LDL and HDL_c to the mutant cells was essentially the same as to normal cells, with equilibrium dissociation constants (K_d) of 2.6×10^{-9} and 0.13×10^{-9} M, respectively. Though the total number of receptors available in the mutant cells from patients with the heterozygous form of familial hypercholesterolemia was only 50% of normal cells, the ratio of LDL to HDL_c bound at receptor saturation remained 4:1. In another mutant cell strain (J. D.) that cannot internalize receptor-bound lipoprotein, the higher affinity of HDL_c for LDL receptors was again demonstrated with K_d values similar to those in normal cell strains. The 4:1 ratio of LDL to HDL_c binding also held. To explore the concept of multiple receptor binding further, we treated normal fibroblasts with paraformaldehyde, a process known to prevent internalization but not binding of LDL. The K_d values for LDL for both treated and untreated cells were identical. This was also true for the K_d values of HDL_c. Furthermore, the expected number of HDL_c particles were bound but only one-half the expected number of LDL particles were bound, giving a 2:1 ratio of LDL bound to normal cells compared with treated cells. These results are consistent with ultrastructural data which indicate that the LDL receptors exist as microaggregates in their native state. When LDL are bound, the receptors migrate to allow individual receptor interaction with LDL. However, paraformaldehyde treatment may cross-link the receptors and prevent lateral receptor migration, thus reducing the number of LDL particles bound. Since apo-E HDL_c normally bind to multiple receptors or clusters of receptors, paraformaldehyde fixation has little effect on their binding. These studies further support the concept that apo-E HDL_c bind to multiple cell surface receptors.

Low-density lipoproteins (LDL)[1] and certain cholesterol-induced atypical high-density lipoproteins (HDL_c) bind to specific lipoprotein receptors on the surface of normal human fibroblast cells in culture and are subsequently internalized and degraded (Brown & Goldstein, 1976a; Mahley et al., 1978; Mahley & Innerarity, 1978). The HDL_c induced in dogs and swine by cholesterol feeding contain high concentrations of the arginine-rich apoprotein (apo-E) that mediates their receptor binding (Innerarity & Mahley, 1978), just as the B apoprotein (apo-B) mediates the binding of LDL (Mahley et al., 1977a; Shireman et al., 1977).

Significant differences have been noted between LDL containing apo-B and HDL_c containing apo-E in their interaction with the cell surface binding sites. Competitive binding studies by Innerarity & Mahley (1978) performed at 4 °C showed that apo-E HDL_c, an HDL_c that contains only the E apoprotein, exhibit a 100-fold greater ability than LDL to displace [^{125}I]LDL from high-affinity sites on the cell surface of normal human fibroblasts. This enhanced binding of apo-E HDL_c was confirmed by Pitas et al. (1979) in equilibrium and kinetic studies using iodinated LDL and apo-E HDL_c. Also, 4 times as many LDL as apo-E HDL_c particles bound to the cells at maximum receptor occupancy, so fewer apo-E HDL_c particles were required to saturate the receptor sites. Since it has been demonstrated by Innerarity & Mahley (1978) and Pitas et al. (1979) that LDL can completely displace [^{125}I]-apo-E HDL_c from high-affinity binding sites and likewise that apo-E HDL_c totally, competitively inhibited the binding of [^{125}I]LDL, the most feasible explanation is that apo-E HDL_c bind to four apo-B,E receptors.

In these studies, we examined two alternative explanations for these observations to confirm and extend the concept of the multiple receptor binding of apo-E HDL_c. The first was that the binding of apo-E HDL_c to one receptor suppresses three other receptors, rendering them unavailable for either LDL or apo-E HDL_c binding. This possibility was studied by binding apo-E HDL_c to the receptor, removing them, and then comparing the ability of treated cells and control cells to bind [^{125}I]LDL.

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[1] Abbreviations used: HDL, high-density lipoproteins; LDL, low-density lipoproteins; HDL_c, cholesterol-induced HDL containing apoproteins A-I and E; apo, apoproteins; apo-E HDL_c, HDL_c containing predominantly apo-E; DME medium, Dulbecco's modified Eagle's medium; FH, familial hypercholesterolemia; K_d, equilibrium dissociation constant.

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The second possibility was that the apo-E HDL_c and LDL bind to adjacent receptors and that the binding of one blocks the binding of the other. This was examined by using cell strains with genetic mutations in the LDL receptor locus. It was reasoned that if LDL and apo-E HDL_c bound to the same receptor, any mutation that changed LDL binding would cause a proportional change in apo-E HDL_c binding. However, if the apo-E HDL_c were binding to a separate, adjacent receptor, then a mutation in the receptor that binds LDL would not affect apo-E HDL_c binding. We used two mutant fibroblast cell strains to investigate this possibility. The first strain of fibroblast cells binds, internalizes, and degrades LDL at about half the normal rate. This cell strain was from patients with the heterozygous form of familial hypercholesterolemia (Goldstein & Brown, 1977). The second mutant cell strain, referred to as the J. D. strain, has receptors that also bind half the normal amount of LDL but do not internalize it (Goldstein et al., 1977; Brown & Goldstein, 1976b).

We used one additional approach. Because paraformaldehyde treatment renders fibroblasts incapable of internalizing bound LDL, we treated a series of fibroblast cells with paraformaldehyde and analyzed the effect on LDL and apo-E HDL_c binding.

Experimental Procedures

Materials. Dulbecco's phosphate-buffered saline (Catalog No. 450-1300), trypsin–EDTA solution (Catalog No. 610-5400), fetal calf serum, Dulbecco's modified Eagle's medium (Catalog No. 430-2100), potassium penicillin G, and streptomycin sulfate were purchased from GIBCO (Grand Island, NY). Sodium [^{125}I]iodide (carrier free) in NaOH and Bolton and Hunter reagent [*N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]-iodophenyl)propionate] were obtained from Amersham/Searle (Arlington Heights, IL). Paraformaldehyde and other reagent grade chemicals were purchased from Fisher Scientific Co. (Fairlawn, NJ). All plasticware for tissue culture was obtained from Falcon Plastics. Reagent grade type-5 polyphosphate (Catalog No. 55878) and practical grade polyphosphate (Catalog No. P8385) were obtained from Sigma (St. Louis, MO).

Plasma Lipoprotein Isolation and Labeling. Human plasma LDL (d = 1.02–1.05 g/cm^3) were isolated by sequential ultracentrifugation from a fasted male subject. Canine HDL_c (d = 1.02–1.063 g/cm^3) and apo-E HDL_c (d = 1.006–1.02 g/cm^3) were obtained from foxhounds fed a semisynthetic diet of cholesterol and hydrogenated coconut oil (Mahley et al., 1977b) and were isolated by a combination of ultracentrifugation and Geon-Pevikon electrophoresis (Mahley & Weisgraber, 1974). The lipoproteins were characterized by paper electrophoresis (Mahley et al., 1974, 1975), and each migrated as a single distinct band. The human LDL migrated with β mobility, and the canine HDL_c or apo-E HDL_c migrated with α-2 mobility. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed that human LDL contained predominantly the B apoprotein, HDL_c isolated at d = 1.02–1.063 g/cm^3 contained mainly the A-I and E apoproteins, and HDL_c isolated at d = 1.006–1.02 g/cm^3 usually contained only the E apoprotein (Mahley et al., 1977b). The chemical compositions of human LDL and canine apo-E HDL_c were similar to that previously reported (Mahley et al., 1977b; Innerarity & Mahley, 1978; Sherrill et al., 1980). Human lipoprotein-deficient serum was prepared by centrifugation in a 60 Ti rotor (Beckman, Inc.) at d = 1.215 g/cm^3 for 48 h at 59000 rpm. Human LDL were iodinated by the iodine monochloride method as described by Bilheimer et al. (1972). Canine apo-E HDL_c were iodinated by the Bolton–Hunter procedure as previously described (Innerarity et al., 1979). Apo-E HDL_c iodinated by the iodine monochloride procedure damaged the lipoproteins and resulted in high nonspecific binding to human fibroblast cells in culture. LDL, however, iodinated by the Bolton–Hunter procedure were indistinguishable from LDL iodinated by the iodine monochloride procedure when both were used in 4 °C binding studies. The specific activities of human [^{125}I]LDL and [^{125}I]apo-E HDL_c were usually 150–250 and 250–900 cpm/ng of protein, respectively. An average of 2.5% of the radioactivity in the human [^{125}I]LDL and 9.0% of the radioactivity in the canine [^{125}I]apo-E HDL_c were soluble in chloroform–methanol (2:1 v/v).

Cells. Normal human fibroblasts were established from a preputial specimen from a healthy infant. Two mutant fibroblast cell strains of heterozygous familial hypercholesterolemia, GM 376 and GM 483 ($+/R^{b^0}$), were purchased from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). A third mutant ($R^{b^0}/R^{b+,i^0}$) cell strain (J. D.) that binds but does not internalize LDL was a gift from Drs. Joseph L. Goldstein and Michael S. Brown.

All cells were grown in monolayers in 75-cm^2 flasks. On day 1, normal fibroblasts were split in a 1:4 ratio; all other cell strains were split 1:3. The cultures were grown in a humidified incubator (8% CO_2) at 37 °C on Dulbecco's modified Eagle's medium (DME medium) supplemented with 10% fetal calf serum, 3.7 mg/mL $NaHCO_3$, 100 units of potassium penicillin G, and 100 μg/mL streptomycin sulfate. On day 3, 4, or 5, cells were dissociated from stock flasks with a 0.05% trypsin–0.02% EDTA solution and plated in 35-mm (3×10^4 cells) or 60-mm (9×10^4 cells) petri dishes. They were used in experiments 1 week later.

Binding. Forty-eight hours before the beginning of an experiment, the cells were washed once with DME medium containing 5% lipoprotein-deficient human serum, and then 3 or 1 mL of DME medium containing 10% lipoprotein-deficient serum was added to each 60- or 35-mm dish, respectively. The binding assay at 4 °C using 60-mm plates was performed exactly as described by Pitas et al. (1979). For the binding assay using cells grown in 35-mm petri dishes, all reagents were reduced appropriately. Cells grown in 35-mm petri dishes were incubated for 3 h on ice with 0.68 mL of DME medium containing 10% lipoprotein-deficient serum and iodinated LDL or iodinated apo-E HDL_c. Receptor-bound lipoprotein (specific binding) was calculated at each lipoprotein concentration by subtracting from the total radioactivity bound the amount not displaced by high concentrations of lipoproteins. Human LDL (500 μg/mL) were used for the measurement of the specific binding of [^{125}I]LDL, and cholesterolemic canine HDL_c (d = 1.02–1.063 g/cm^3; 100 μg/mL) were used for the measurement of the specific binding of [^{125}I]apo-E HDL_c. The radioactivity remaining after saturation of the receptor with nonradioactive lipoproteins represented the nonspecific component and was related linearly to the concentration of the iodinated lipoproteins. At the highest concentration employed, it constituted less than 12% of the total bound radioactivity.

Polyphosphate was used to release [^{125}I]apo-E HDL_c from the cell surface receptors of cultured fibroblasts. The procedure was exactly as described by Goldstein et al. (1976), except polyphosphate was substituted for heparin. Maximum release at 4 °C was at concentrations of 30 mg/mL for apo-E HDL_c and 10 mg/mL for LDL. In the initial experiments, reagent grade type-5 was used. Subsequent experiments demonstrated that the much less expensive practical grade was just as effective, and it was used in these studies. Paraformaldehyde

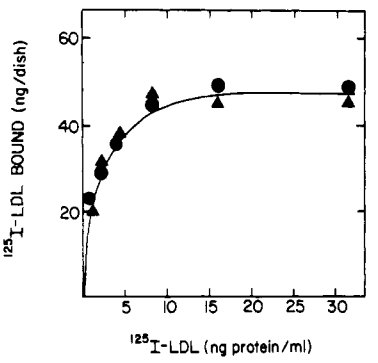

FIGURE 1: Effect of prior binding of apo-E HDL_c and polyphosphate release on receptor binding of human [^{125}I]LDL. [^{125}I]LDL bound to control cells (●) and to cells which had previously bound apo-E HDL_c (▲). The cells were cooled for 30 min at 4 °C, and the medium was removed from each dish. Then 2 mL of DME medium (25 mM Hepes buffer, pH 7.4) containing 10% human lipoprotein-deficient serum was added either alone (control) or with 0.22 μg/mL [^{125}I]apo-E HDL_c (70 cpm/ng). After 1 h at 4 °C, each dish was washed by the standard method. The plates were incubated for 1 h at 4 °C with the last phosphate-buffered saline wash, which contained polyphosphate (30 mg/mL). This wash was removed. Each dish was then washed 3 times with cold phosphate-buffered saline and then incubated at 4 °C with 2 mL of medium containing 10% human lipoprotein-deficient serum, 25 mM Hepes buffer (pH 7.4), and the indicated amounts of [^{125}I]LDL. After a 3-h incubation at 4 °C, each dish was washed by the regular procedure and the amount of receptor-bound [^{125}I]LDL was determined. The mean cellular protein was 0.33 mg/dish.

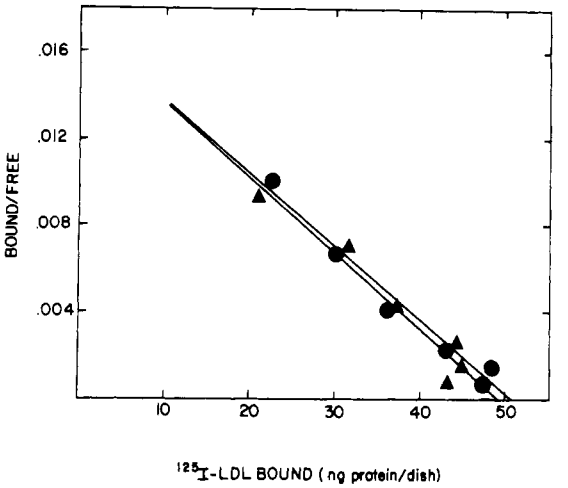

FIGURE 2: Scatchard plot for binding of human [^{125}I]LDL to control cells (●) and to cells which had previously bound apo-E HDL_c (▲). Bound/free = [lipoprotein bound (nanograms of protein/dish)]/[lipoprotein free in the medium (nanograms of protein/2 mL)]. These data are shown in Figure 1.

fixation of normal human fibroblast cells was performed as described by Brown et al. (1976).

Analysis. The equilibrium dissociation constant (K_d) was determined by plotting the ratio of receptor-bound to free lipoprotein vs. receptor-bound lipoprotein as described by Scatchard (1949). The maximum amount of lipoprotein bound at receptor saturation (nanograms of lipoprotein protein per dish) was obtained from the x intercept. Molecular weights of 3.6×10^6 (of which 15% is protein) for apo-E HDL_c (Innerarity & Mahley, 1978) and 3×10^6 (of which 20% is protein) for LDL (Fisher et al., 1972) were used to convert from nanograms of lipoprotein to moles.

Results

Receptor Suppression Experiments. To test the hypothesis that apo-E HDL_c binding to one receptor suppressed three other receptors, we removed apo-E HDL_c bound to the cells and then determined the ability of the cells to bind LDL. To remove apo-E HDL_c from cell surface receptors, we used polyphosphate. Heparin and dextran sulfate, which are commonly employed to remove surface-bound LDL (Goldstein et al., 1976), were ineffective in releasing surface-bound apo-E HDL_c. A number of other reagents, previously used to release [^{125}I]LDL from the cell surface, were tested (Goldstein et al., 1976). Polyphosphate proved most suitable. In fact, more than 86% of the [^{125}I]apo-E HDL_c bound to the cells at 4 °C could be released by treatment with 30 mg/mL polyphosphate. This allowed us to determine if apo-E HDL_c binding irreversibly changed the affinity or capacity of receptors to bind [^{125}I]LDL. As shown in Figure 1, cells that previously had bound apo-E HDL_c could bind the same amount of human [^{125}I]LDL as control cells. Scatchard (1949) analysis of the data in Figure 1 showed that even after binding and polyphosphate release of HDL_c, the cells bound normal amounts of [^{125}I]LDL (49 ng/plate vs. 51 ng/plate for control cells) with similar affinity ($K_d = 2.5 \times 10^{-9}$ M vs. 2.4×10^{-9} M for control cells, Figure 2). Thus, the receptors were unaffected by prior binding of HDL_c and polyphosphate treatment.

Receptor Blocking Experiments. The hypothesis that apo-E and apo-B receptors were distinct sites, such that apo-E HDL_c binding might block apo-B sites, would predict that mutations in the LDL receptor (apo-B) would not affect the apo-E binding site. Fibroblasts with a mutation at the LDL receptor locus were used to test this possibility. The first fibroblast strains studied had been derived from patients with the heterozygous form of familial hypercholesterolemia. These cells have been shown by Brown & Goldstein (1974) to bind about half as many molecules as normal cells.

Binding experiments were conducted at 4 °C to determine if apo-E HDL_c binding was reduced to the same extent in these mutant fibroblast cells as LDL binding was. Scatchard analysis of one experiment showed that the affinity of LDL for the abnormal cells (shown by the slope) was almost identical with that of normal cells (Figure 3A). The enhanced affinity of apo-E HDL_c was similar for both the normal and the mutant cells (Figure 3B). At receptor saturation, the binding of apo-E HDL_c remained in the same proportion to LDL binding, but both reflected the reduced number of receptors. Data for the binding of apo-E HDL_c and LDL to normal and mutant fibroblasts are shown in Table I. The K_d for LDL binding was similar in all cell strains and almost identical with the value previously reported by Pitas et al. (1979) (2.8×10^{-9} M). Apo-E HDL_c bound with a much higher affinity than LDL, as previously shown (Pitas et al., 1979). As with LDL, the K_d for the binding of apo-E HDL_c was similar for all cell strains (Table I). The maximum amount of [^{125}I]LDL bound (and therefore the number of LDL receptors) in the mutant cell strains averaged 40% of the amount bound in normal cells (Table I). In both the normal and mutant cell strains, an average of 3.6 times more LDL than apo-E HDL_c was bound to the cells at receptor saturation.

Additional binding studies at 4 °C were done to determine if apo-E HDL_c would bind to multiple receptors in the mutant J. D. cell strain. Fibroblasts of this strain bind but do not internalize LDL (Goldstein et al., 1977). Binding of both [^{125}I]LDL (Figure 4A) and [^{125}I]apo-E HDL_c (Figure 4B) was reduced in proportion to the reduction in the number of receptors. As illustrated by the slope of the Scatchard plots, [^{125}I]LDL (Figure 4C) and [^{125}I]apo-E HDL_c (Figure 4D) bound with similar affinity to both J. D. cell receptors and the normal cell receptors. The results of four experiments con-

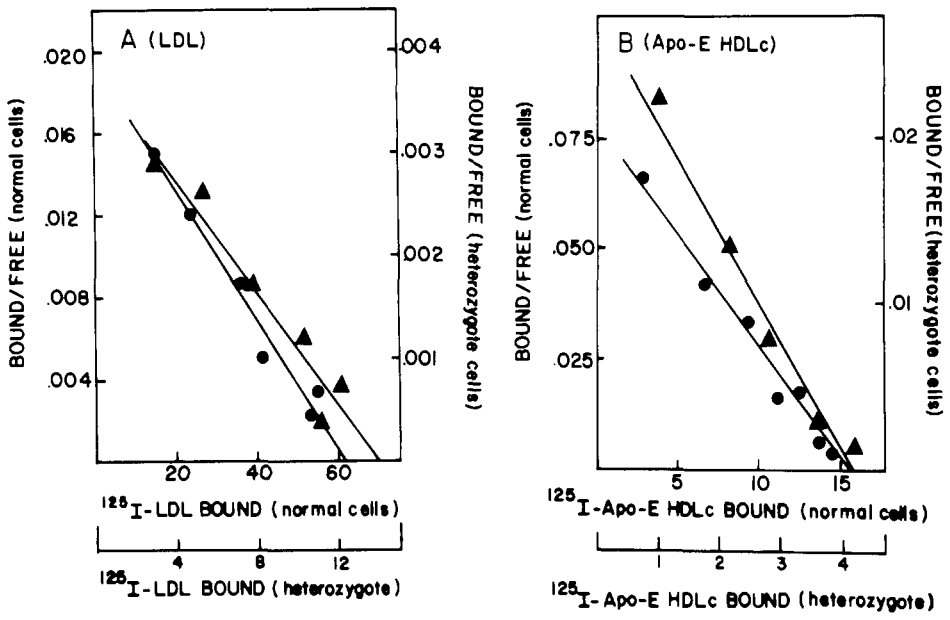

FIGURE 3: Scatchard plot for binding of [^{125}I]LDL (A) and [^{125}I]apo-E HDL$_c$ (B) to normal (●) and heterozygous FH (▲) human fibroblasts. Bound/free = [lipoprotein bound (nanograms of protein/dish)]/[lipoprotein free in the medium (nanograms of protein/2 mL)]. Note that both the ordinate and abscissa for heterozygous FH cells differ from those of normal cells. The monolayers were incubated for 3 h at 4 °C with the iodinated lipoproteins at the concentrations indicated. Data have been corrected for nonspecific binding (see Experimental Procedures). Each point represents the average of duplicate determinations. The mean cellular protein in the 60-mm dishes was 0.35 mg/dish (8.3×10^5 cells/dish) for the mutant and 0.45 mg/dish (1×10^6 cells/dish) for the normal cells. The specific activities were 243 and 254 cpm/ng of protein for [^{125}I]LDL and [^{125}I]apo-E HDL$_c$, respectively.

Table I: Parameters for the Binding of [^{125}I]LDL and [^{125}I]HDL$_c$ to the Receptors of Normal and Two Heterozygous Familial Hypercholesterolemic Cell Strains

	LDL			apo-E HDL$_c$		
parameter	normal	GM 376	GM 483	normal	GM 376	GM 483
K_d,[a] M ($\times 10^{-9}$)						
expt 1[b]	2.4	2.7	3.4	0.61	0.41	0.50
expt 2	2.6	2.9	3.0	0.18	0.28	0.16
av	2.5	2.8	3.2	0.40	0.34	0.33
bound[c] ($\times 10^4$)						
expt 1	9.1	6.3	2.6	2.60	1.30	0.70
expt 2	5.8	2.1	1.7	1.60	0.78	0.57

LDL/HDL$_c$[d]		
normal	GM 376	GM 483
3.6	3.8	3.4

[a] Equilibrium dissociation constant. [b] Most apo-E HDL$_c$ preparations contained only trace amounts of apoproteins other than apoprotein E. This preparation (experiment 1), however, contained ~10% A-IV, ~5% A-I, and ~4% C's (as estimated from polyacrylamide gel electrophoresis). The lower affinity for this apo-E HDL$_c$ may have been due to these additional apoproteins. [c] Particles of lipoprotein bound per cell. Ratio calculated from the number of particles bound per cell, determined as follows: (g of protein/dish)/(M_r of lipoprotein × % lipoprotein protein) = moles/dish; (moles/dish)(Avogadro's number) = molecules (particles)/dish; (particles/dish)/(cells/dish) = particles bound/cell. [d] Ratio of LDL particles bound per cell to apo-E HDL$_c$ particles bound per cell; average of two experiments. In experiment 1, all cell strains were placed at 9×10^4 cells/dish on day 1. On day 7, the day of the experiment, the cell count was 8.6×10^5, 4.0×10^5, and 7.0×10^5 for normal, GM 376, and GM 483, respectively. In the second experiment, to achieve more nearly the same cell confluency for all three cell strains on the day of the experiment, different numbers of cells were plated. On day 0, the cells were plated at 7×10^4, 2.5×10^5, and 2.0×10^5, and on day 7, the cell number had increased to 1.07×10^6, 7.1×10^5, and 8.3×10^5 for normal, GM 376, and GM 483 cells, respectively.

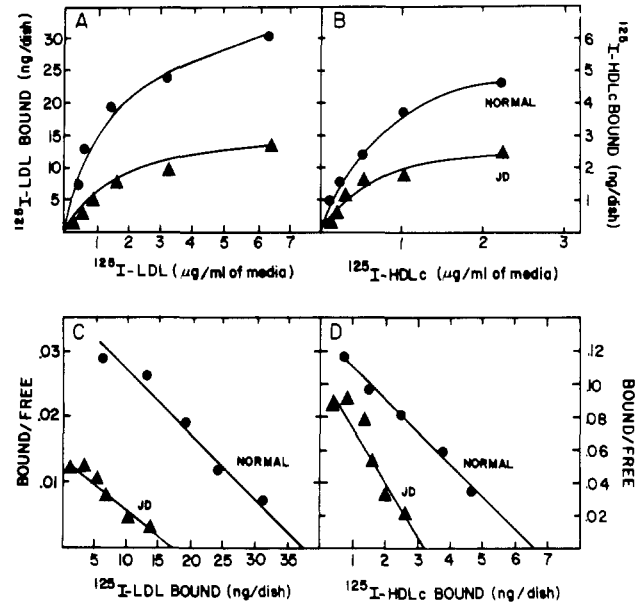

FIGURE 4: Concentration-dependent binding of human [^{125}I]LDL (A), with the resulting Scatchard plot (C), and of canine [^{125}I]apo-E (B), with the resulting Scatchard plot (D), to normal (●) and J. D. (▲) fibroblast cell strains. The cells in 35-mm dishes were incubated for 3 h at 4 °C with the iodinated lipoproteins at the concentrations indicated. Data have been corrected for nonspecific binding. Each point represents the average of duplicate determinations. The specific activities were 156 and 828 cpm/ng of protein for [^{125}I]LDL and [^{125}I]apo-E HDL$_c$, respectively. The mean cellular protein in the 35-mm dishes was 0.13 mg/dish (4.1×10^5 cells/dish) for the J. D. cells and 0.12 mg/dish (3.7×10^5 cells/dish) for the normal cells.

firmed that the affinity of LDL for J. D. cell receptors (K_d = 2.7×10^{-9} M) was almost identical with their affinity for normal receptors (K_d = 2.9×10^{-9} M). At receptor saturation, the ratio of LDL to HDL$_c$ particles bound was similar in both the J. D. cells and normal cells (Table II).

Receptor Migration. Anderson et al. (1977) suggested that the defect in J. D. cells may be an inability of the receptors to cluster and migrate to the coated pits where they could be

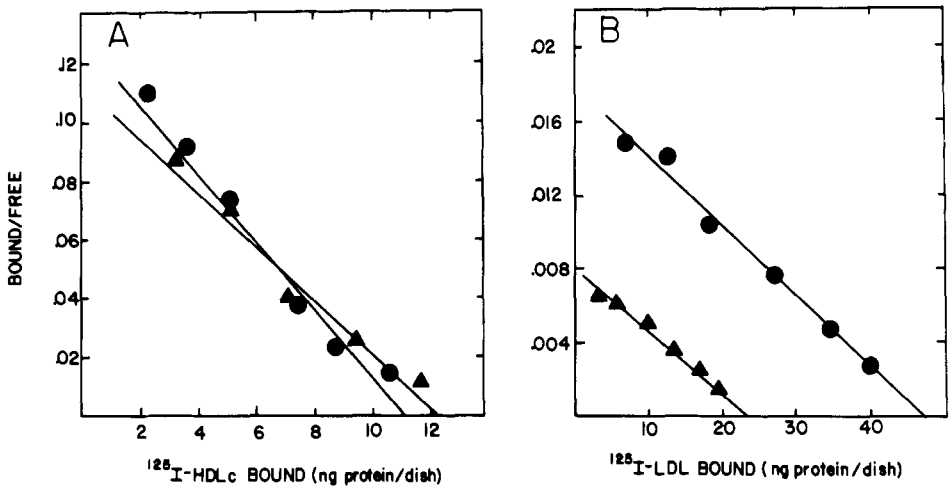

FIGURE 5: Scatchard plot of the binding of canine [^{125}I]apo-E HDL$_c$ (A) and [^{125}I]LDL (B) to normal cells (●) and to normal cells treated with paraformaldehyde (▲). On the day of the experiment, cell monolayers were cooled for 30 min on ice, the medium from each dish was removed and the cells were washed once with phosphate-buffered saline (PBS) and then incubated for 1 h with PBS or with PBS containing 1.6% paraformaldehyde. After incubation on ice (4 °C) for 1 h, each monolayer was washed 4 times with (3 mL/wash) PBS containing 2 mg/mL bovine serum albumin. Each dish received 2 mL of medium containing 10% human lipoprotein-deficient serum, 25 mM Hepes buffer (pH 7.4), and the indicated amounts of [^{125}I]LDL or [^{125}I]apo-E HDL$_c$. After a 3-h incubation at 4 °C, each dish was washed by the standard technique (Pitas et al., 1979) and the amount of receptor-bound radioactivity was determined. Each value represents the average of duplicate determinations. The specific activities were 168 and 889 cpm/ng of protein for [^{125}I]LDL and [^{125}I]apo-E HDL$_c$, respectively. The mean cellular protein in the 60-mm dishes was 0.42 mg/dish.

Table II: Parameters for the Binding of [^{125}I] LDL and [^{125}I] Apo-E HDL$_c$ to the Receptors of J. D. Cell Strains

parameter	LDL	apo-E HDL$_c$
K_d,[a] M	$(2.7 \pm 0.6) \times 10^{-9}$	$(0.05 \pm 0.019) \times 10^{-9}$
LDL/HDL$_c$[b]	4.5 ± 1.3	

[a] Equilibrium dissociation constant ± SD. Data were derived from Scatchard plots of equilibrium binding data of four separate experiments performed at 40 °C. [b] Ratio of LDL particles bound per cell to apo-E HDL$_c$ particles bound per cell ± SD.

internalized. Binding experiments were conducted at 37 °C with J. D. cells to determine if apo-E HDL$_c$ facilitate receptor migration to the coated pits and result in more internalization and degradation of apo-E HDL$_c$ than LDL. Our results resembled those of Brown & Goldstein (1976b) for [^{125}I]LDL; J. D. cells internalized and degraded much less [^{125}I]LDL than normal cells. Analogously, J. D. cells took up less apo-E HDL$_c$ than did normal cells, indicating that apo-E HDL$_c$ binding to receptor clusters did not facilitate uptake. When normal cells were incubated for 5 h at 37 °C with 6.4 μg/mL [^{125}I]LDL, 76 times more [^{125}I]LDL was internalized and degraded than was bound at the cell surface. Under the same conditions, the ratio of internalized and degraded to bound for J. D. cells was 4.8. Likewise, with normal cells, 76 times more [^{125}I]apo-E HDL$_c$ was internalized and degraded than was bound to the receptors, compared to a ratio of 7.0 for J. D. cells.

To study another atypical binding situation, we fixed normal fibroblast monolayers with paraformaldehyde. Such fixed cells are known to bind [^{125}I]LDL (Brown et al., 1976). To determine if they would also bind apo-E HDL$_c$, we incubated paraformaldehyde-treated cells with increasing concentrations of [^{125}I]apo-E HDL$_c$ and carried out the standard 4 °C binding assay. Paraformaldehyde fixation of the cells had little effect on apo-E HDL$_c$ binding (Figure 5A). A maximum of 11 ng of apo-E HDL$_c$ protein per plate bound to the control cells compared with 12 ng/plate for paraformaldehyde-treated cells. The affinity of [^{125}I]apo-E HDL$_c$ for the treated cells also resembled control values ($K_d = 0.078 \times 10^{-9}$ M for control vs. 0.1×10^{-9} M for treated cells). By contrast, treated cells bound only half as much [^{125}I]LDL as the control cells (23 vs. 47 ng/plate), although K_d values were nearly identical for the control and fixed cells, 2.2×10^{-9} and 2.4×10^{-9} M, respectively (Figure 5B). Thus, for LDL binding, the fixed cells behaved like a mutant cell strain in that only half the total number of receptors were functional, whereas for apo-E HDL$_c$ the fixed cells had the full complement of receptors. The observation that more [^{125}I]LDL bound to control cells than to fixed cells could have resulted from internalization of receptor-bound LDL by the control cells at 4 °C. This possibility was ruled out by repeating the binding studies and measuring only the surface-bound lipoproteins after they had been released by polyphosphate treatment. These results revealed that apo-E HDL$_c$ bound equally to control and fixed cells (11.5 ng/plate for control vs. 12.0 ng/plate for treated cells), but less than half as much LDL bound to the fixed cells as to the control cells (24.1 ng/plate for treated vs. 59.0 ng/plate for control cells).

These experiments suggested that in paraformaldehyde-treated cells, apo-E HDL$_c$ bound to the normal complement of receptors, while LDL bound only to about half the total number of apo-B,E receptors. If this interpretation were correct, then in competition experiments less LDL would be needed to displace [^{125}I]apo-E HDL$_c$ from the apo-B,E receptors of paraformaldehyde-treated cells than from the receptors of normal cells. That is, the clusters of receptors that bind HDL$_c$ and that were fixed as clusters by paraformaldehyde treatment could not be lateralized when LDL competed with HDL$_c$; therefore, the treated cells would bind less than the normal amount of LDL (see Discussion). In the 4 °C experiment shown in Figure 6, one-third as much LDL was needed to displace an equivalent amount of [^{125}I]apo-E HDL$_c$ from the treated cells as from the nontreated control cells. Note that when the data were plotted on a logit-log scale (inset, Figure 6), parallel curves were obtained for the competitive binding experiment with normal and paraformaldehyde-treated cells. The parallel curves indicate that the receptor–lipoprotein interactions were the same in the treated and control cells. If the receptors had been partially altered by the fixative, the slopes (affinities) would have differed for control and treated cells on both the Scatchard plot (direct binding experiments)

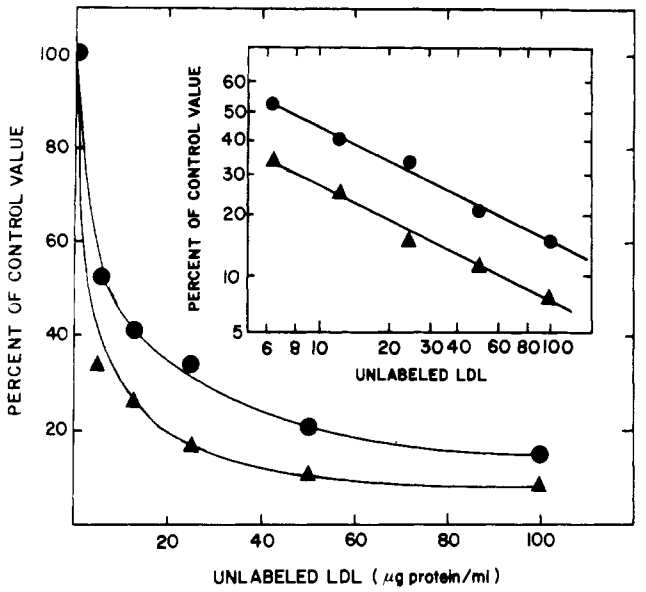

FIGURE 6: Competitive displacement of ^{125}I-labeled apo-E HDL_c by human LDL from normal (●) and paraformaldehyde-treated human fibroblasts (▲). Half of the cells were fixed as described in the legend to Figure 5; the other half received the same treatment except that the paraformaldehyde was omitted. The [^{125}I]apo-E HDL_c (0.1 μg/mL; 258 cpm/ng of protein) were added to the dishes in medium containing 25 mM Hepes (pH 7.4), 10% human lipoprotein-deficient serum, and the concentrations of unlabeled LDL indicated; the incubation was for 2 h at 4 °C. The mean cellular protein for the control dishes was 0.31 mg/dish. In the inset, the data were analyzed by logit-log transformation (normal, ●; treated, ▲).

and the logit-log plot (competitive binding experiments). The results could be explained, however, if about half of the receptors were, for some reason, unavailable to bind LDL.

Further evidence supporting the validity of the above experiments includes two 4 °C binding experiments. In the first experiment, similar amounts of LDL were needed to displace [^{125}I]LDL, and in the second experiment, similar concentrations of apo-E HDL_c were needed to displace [^{125}I]apo-E HDL_c in control and paraformaldehyde-treated cells.

Discussion

Previous studies by Pitas et al. (1979) have indicated that while less apo-E HDL_c than LDL bind to fibroblast cells, the apo-E HDL_c bind with a higher affinity. The most feasible interpretation for this higher affinity binding to apo-E HDL_c particles is that each particle binds to multiple apo-B,E receptors (LDL receptors). The studies reported in this paper support this explanation. The possibility that an apo-E HDL_c particle binds to one apo-B,E receptor and suppresses three others was excluded by demonstrating that apo-E HDL_c binding and release did not alter the binding characteristics of apo-B,E receptors; i.e., LDL binding to cells that had previously bound apo-E HDL_c was the same as that binding to control cells. A second possibility, that apo-E HDL_c binds to different and adjacent receptors, was disproved by using two fibroblast cell strains, each with different genetic defects at the LDL receptor locus. If apo-E HDL_c bound to different, adjacent receptors, then a mutation at the LDL receptor locus would not have affected apo-E HDL_c binding.

One of the mutant cell strains studied was derived from patients with the heterozygous form of familial hypercholesterolemia (heterozygous FH). These cells display roughly half as many functional receptors as normal fibroblast cells. The other cell strain (J. D.), like the heterozygous FH strain, has a receptor-negative allele that produces a functionally silent receptor. The other allele at the receptor locus of the J. D. strain codes for receptors which can bind LDL normally but cannot couple the binding and internalization processes. These mutant cell strains bound [^{125}I]LDL with the same affinity as normal human fibroblast receptors. Apo-E HDL_c also bound to the mutant cell strains with the same enhanced affinity previously seen in normal cells (Innerarity & Mahley, 1978; Pitas et al., 1979). Regardless of the cell strain, apo-E HDL_c bound to approximately four apo-B,E receptors, remaining in direct proportion to LDL binding observed in the same cell strains. These studies eliminate the possibility of apo-E HDL_c binding to receptors other than those of the LDL receptor locus. Moreover, cell strains devoid of LDL receptor binding (homozygous FH) did not bind canine apo-E HDL_c (data not shown) or swine HDL_c (Bersot et al., 1976), and J. D. cells did not internalize apo-E HDL_c.

For apo-E HDL_c to bind to multiple receptors, the receptors on the cell surface must cluster to permit contact with the apo-E HDL_c. However, for LDL to bind to individual receptors, the same receptors need to be sufficiently separated (or mobile) to permit each receptor to contact a particle of LDL without steric hindrance. Interestingly, both the receptors for LDL and the cluster of receptors that bound apo-E HDL_c behaved, in equilibrium and kinetic experiments, as single binding sites. That is, the binding of both LDL and apo-E HDL_c to the cell fits a simple biomolecular ligand–receptor model with no evidence of receptor cooperativity, lipoprotein aggregation, or the existence of more than one class of binding sites, e.g., high- and low-affinity binding sites (Pitas et al., 1979).

We suggest that within localized regions of the cell membrane, the apo-B,E receptors exist as microaggregations held together by weak attachments or by substructural proteins. The receptors would not have a rigidly fixed arrangement but would be weakly associated with each other. Because apo-E HDL_c, a lipoprotein about the same size and molecular weight as LDL, can bind to 4 times as many receptors, the receptors must cluster in groups of at least four. Because LDL binds to individual receptors without any evidence of receptor associations (as would be expected if cooperative effects had been seen in kinetic and equilibrium studies), then the association between LDL receptors must be weak, permitting sufficient lateral mobility for individual receptors to bind to LDL particles without steric interference. Anderson et al. (1977) have experimental evidence to support this concept of LDL receptors grouped in an orderly arrangement on the cell surface. By electron microscopy of receptor-bound LDL–ferritin, they observed that 50–80% of the receptors were concentrated in coated pits that constitute only 2% of the cell surface. Moreover, within the coated pits, which appear to be located in linear arrays along the cell surface, the receptors were grouped in clusters of seven to nine (Anderson et al., 1980). In the J. D. cells, the receptors fail to migrate to the coated pits where they can be internalized by endocytosis, but they still appear as clusters, albeit in groups of three or four (Anderson et al., 1980) which is a number still sufficient for multiple receptor binding of apo-E HDL_c. Likewise, Schechter et al. (1979) have shown that receptor-bound epidermal growth factor diffuses laterally in the plane of the membrane and undergoes some type of self-aggregation of receptors to elicit a biological response.

It is reasonable to suggest that paraformaldehyde cross-links adjacent receptors to each other or to other adjacent membrane proteins, thereby inhibiting their lateral movement. Since paraformaldehyde treatment did not change the binding af-

finity of the receptors, we suggest that the structural integrity of individual receptors was not greatly altered. Therefore, because apo-E HDL_c bind to clusters of receptors, fixation of the cells did not significantly alter the number of apo-E HDL_c particles bound. On the other hand, LDL bound with normal affinity to the fixed fibroblasts; however, at saturation of the cell surface receptors, only about half the number of LDL particles were bound compared with the number bound by normal cells. This would be expected if receptors were fixed in clusters and the binding of one LDL particle sterically hindered the binding of another LDL particle to an adjacent receptor. Also, as would be expected, the LDL that did bind to the receptors displayed normal binding affinity.

These studies suggest that receptors exist as microaggregates at 4 °C and that paraformaldehyde treatment inhibits the lateral mobility of the receptors in the plane of the membrane. Our studies strongly support the concept that apo-E HDL_c and LDL bind to the same receptor and that apo-E HDL_c bind to multiple receptors.

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