Specific High-Affinity Binding of High Density Lipoproteins to Cultured Human Skin Fibroblasts and Arterial Smooth Muscle Cells

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ABSTRACT Binding of human high density lipoproteins (HDL, d = 1.063–1.21) to cultured human fibroblasts and human arterial smooth muscle cells was studied using HDL subjected to heparin-agarose affinity chromatography to remove apoprotein (apo) E and B. Saturation curves for binding of apo E-free ^{125}I-HDL showed at least two components: low-affinity nonsaturable binding and high-affinity binding that saturated at ~20 μg HDL protein/ml. Scatchard analysis of high-affinity binding of apo E-free ^{125}I-HDL to normal fibroblasts yielded plots that were significantly linear, indicative of a single class of binding sites. Saturation curves for binding of both ^{125}I-HDL_3 (d = 1.125–1.21) and apo E-free ^{125}I-HDL to low density lipoprotein (LDL) receptor-negative fibroblasts also showed high-affinity binding that yielded linear Scatchard plots. On a total protein basis, HDL_2 (d = 1.063–1.10), HDL_3 and very high density lipoproteins (VHDL, d = 1.21–1.25) competed as effectively as apo E-free HDL for binding of apo E-free ^{125}I-HDL to normal fibroblasts. Also, HDL_2, HDL_3, and VHDL competed similarly for binding of ^{125}I-HDL_3 to LDL receptor-negative fibroblasts. In contrast, LDL was a weak competitor for HDL binding. These results indicate that both human fibroblasts and arterial smooth muscle cells possess specific high affinity HDL binding sites. As indicated by enhanced LDL binding and degradation and increased sterol synthesis, apo E-free HDL_3 promoted cholesterol efflux from fibroblasts. These effects also saturated at HDL_3 concentrations of 20 μg/ml, suggesting that promotion of cholesterol efflux by HDL is mediated by binding to the high-affinity cell surface sites.

INTRODUCTION

An important function of circulating lipoproteins is the transport of lipids between various tissues in the body. The main function of low density lipoprotein (LDL) appears to be the delivery of esterified cholesterol to cells (1). This process is mediated by high affinity binding of LDL to a cell surface receptor specific for apoproteins (apo)[1] B and E, followed by uptake and degradation of the LDL particle. The exact role of high density lipoprotein (HDL) in cholesterol transport remains unknown. There is some evidence that HDL, as well as LDL, binds specifically to steroid hormone-producing tissues to deliver cholesterol for use in hormone synthesis (2–4). It has been postulated that HDL serves to remove unesterified cholesterol from other extrahepatic cells, which is then esterified and either transferred to lower density lipoproteins or carried to the liver where the cholesterol is ultimately excreted in bile ("reverse cholesterol transport") (5). This removal or efflux of cholesterol from extrahepatic cells may be mediated by the binding of HDL to the cells (6). Prior studies of binding of HDL to extrahepatic cells has been confounded by the fact that HDL separated by the usual ultracentrifugal procedures is a heterogeneous group of particles containing, in addition to apo A-I and A-II, apo E, which binds avidly to the LDL receptor (7). In this study, specific high-affinity binding of HDL devoid of apo E (apo E-free HDL) to extrahepatic cells, independent of the LDL receptor, was demonstrated and characterized, and the possible relationship of this binding to cholesterol efflux from cells was assessed.

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[1] *Abbreviations used in this paper*: apo, apoprotein; LDS, lipoprotein-deficient serum.

METHODS

Cells. Normal human skin fibroblasts were grown from explants of punch biopsies of skin from the inner thighs of normal volunteers. LDL receptor-negative fibroblasts (GM488, GM2000) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). Human arterial smooth muscle cells were grown from explants of intimal-medial segments of thoracic aorta obtained during vascular surgery by methods previously described (8). Cells were grown in plastic tissue culture flasks in modified Dulbecco-Vogt medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) containing 10% pooled human serum at 37°C in humidified incubators equilibrated with 5% CO_2, 95% air. For most experiments, cells were trypsinized from stock flasks (two to eight passages) and seeded in 35-mm plastic petri dishes using 2 ml of medium (10% pooled human serum) containing $\sim 5 \times 10^4$ cells. Cells were grown in medium containing 10% fetal calf serum (fibroblasts) or 10% pooled human serum (smooth muscle cells) and used just before reaching confluency, usually 5–7 d after plating. The growth medium was changed every 2–3 d. Unless indicated otherwise, each data point for the figures and tables represents the mean of determinations on duplicate dishes.

Lipoproteins and lipoprotein-deficient serum (LDS). Lipoproteins were isolated from human serum by sequential ultracentrifugation (9). The lipoprotein fractions were separated according to densities as follows: low density (LDL, d = 1.019–1.063); high density (HDL, d = 1.063–1.21); high density$_2$ (HDL_2, d = 1.063–1.100); high density$_3$ (HDL_3, d = 1.125–1.21); and very high density (VHDL, d = 1.21–1.25). LDL and HDL were iodinated with ^{125}I by the McFarlane (10) monochloride procedure as modified for lipoprotein. The specific activity for both ^{125}I-LDL and ^{125}I-HDL was $\sim 2.0 \times 10^5$ cpm/μg of protein. LDS was prepared by centrifugation of pooled human serum at d = 1.25 at 176,000 g for 28 h. The bottom fraction was isolated and recentrifuged. Apo A-I, A-II, and B were determined by radioimmunodiffusion techniques (11, 12).

Apo E- and apo B-free HDL and sterol-depleted HDL. Fractions of HDL that were devoid of apo E were prepared by affinity column chromatography. HDL was passed through a heparin-agarose affinity column, essentially as described by Weisgraber and Mahley (13), prepared by the method of Mitchell et al. (14). HDL was added to the column in 0.005 M imidazole buffer (pH 6.5) containing 0.015 M NaCl. After the unbound HDL fraction was eluted from the column, the bound HDL was removed by increasing the NaCl concentration to 1.0 M. Elution of protein was monitored by measurement of absorbance at 280 nm. To ascertain that apo E and B were completely removed from HDL, the unbound fraction was tested for its ability to compete for ^{125}I-LDL binding to fibroblasts. It was assumed that these HDL preparations were devoid of apo E and B if HDL could not displace ^{125}I-LDL from the cell surface at HDL protein concentrations of 100-fold greater than the LDL protein concentration.

Lipid-depleted HDL_3 was prepared by heptane extraction as described by Krieger et al. (15), except that the extractions were performed at 0° rather than at −10°C, and no lipids were added to the extracted lipoprotein. This procedure removed >60 and 80% of the HDL_3 unesterified and esterified cholesterol content, respectively, without affecting the phospholipid content or apoprotein composition.

Binding of ^{125}I-LDL. For measurement of binding of labeled LDL to cells, cells preincubated in the indicated media were chilled to 0°C for 20 min. The preincubation medium was aspirated and discarded. The cell layers were washed two times with ice-cold phosphate-buffered saline (PBS) (0.2 g/liter KCl, 0.2 g/liter KH_2PO_4, 8.0 g/liter NaCl, 2.16 g/liter $Na_2HPO_4 \cdot 7H_2O$). Serum-free medium containing 10 mM HEPES buffer (pH 7.4) plus 2 mg/ml albumin with ^{125}I-LDL was added to the dishes (1 ml/dish) and the cells were incubated at 0°–4°C for 2 h. After 2 h the incubation medium was removed and discarded. The dishes were then rapidly washed four times with 2.0 ml of cold PBS containing 2 mg/ml albumin (wash medium), followed by two 10-min washes with the same medium, and one wash with cold PBS. Each dish then received 2.0 ml of cold 10 mM HEPES buffer (pH 7.4) containing 9.0 mg/ml NaCl and 4.0 mg/ml dextran sulfate. After 1 h, the dextran sulfate medium was removed and the cells were dissolved in 0.1 M NaOH for protein determinations. The amount of radioactivity released by dextran sulfate was assumed to represent receptor-bound ^{125}I-LDL (16, 17).

Binding of ^{125}I-HDL. After cells were grown to confluence, the dishes were washed with sterile PBS once and then were preincubated for 12–18 h at 37°C in serum-free medium plus 2 mg/ml albumin. This preincubation was performed to allow time for lipoproteins to dissociate from cell surface binding sites and for cell cholesterol metabolism to equilibrate. Cholesterol efflux is negligible in the presence of serum-free medium (17). Cells were then washed once at room temperature with 2.0 ml wash medium and chilled at 4°C after the addition of another 2.0 ml wash medium. The cold wash was then removed and serum-free medium containing 2 mg/ml albumin, 10 mM HEPES (pH 7.4), and ^{125}I-HDL was added to the dishes (1 ml/dish). The cells were incubated at 0°–4°C for 2 h. After 2 h, the incubation medium was removed and discarded. The dishes were then subjected to the same wash procedure as with LDL binding. After the final wash with PBS, the amount of ^{125}I-HDL bound was assessed by two separate procedures. For the first procedure, each dish received 1.0 ml medium containing 0.05% trypsin plus EDTA and the dishes were incubated at 37°C for 10 min. The trypsin was then neutralized by addition of medium containing serum and the cells were pelleted by centrifugation. The amount of ^{125}I-radioactivity in the supernatant was assumed to represent bound ^{125}I-HDL. The washed pellet was then dissolved in 0.1 N NaOH and an aliquot was counted for ^{125}I-radioactivity, assumed to represent internalized ^{125}I-HDL, and another aliquot was used for protein determinations as described by Lowry et al. (18). For the second procedure, cells were washed once again with cold PBS and 1.0 ml of 0.1 N NaOH was added directly to the washed monolayer. Part of the NaOH digest was used for protein determinations and part was used to determine the amount of cell-associated radioactivity.

The saturation kinetics for HDL binding were evaluated from data obtained for binding studies in the presence of increasing concentrations of ^{125}I-HDL. The amount of saturable or specific binding at each HDL concentration was estimated by subtraction of the amount of nonspecific HDL bound from the total HDL bound. The nonspecific component was determined from either (*a*) the slope of the saturation curve that appeared to be linear at HDL concentrations above saturation of specific binding sites, or (*b*) values for ^{125}I-HDL binding obtained in the presence of at least 50-fold excess of unlabeled HDL. The ratios of bound to free ^{125}I-HDL were plotted against bound HDL according to the method of Scatchard (19).

Internalization, degradation, or dissociation of bound ^{125}I-HDL. To determine the fate of prebound HDL, apo E-free ^{125}I-HDL was first bound to the cell surface at 0°C

and the cells were thoroughly washed by the procedure described for the HDL binding assay. Each dish then received ice-cold serum-free culture medium containing 2 mg/ml albumin plus or minus unlabeled apo E-free HDL. One set of dishes was then warmed to 37°C while another set of dishes was maintained at 0°–4°C. After the indicated time, the medium was collected and treated with TCA (final concentration of 8%). The ^{125}I-radioactivity in the acid-insoluble (undegraded HDL) and acid-soluble (degraded HDL) was measured. The cells were rapidly washed once with PBS and harvested by the trypsinization procedure described for the HDL binding assay. The harvested cells were chilled to 0°C, washed once with ice-cold PBS and dissolved in 0.1 N NaOH. The trypsin medium and NaOH digest were counted for ^{125}I-radioactivity to determine the quantities of HDL bound and internalized.

Electrophoresis of bound and dissociated ^{125}I-HDL. Apo E-free ^{125}I-HDL that was released from cell surface binding sites was characterized by one-dimensional gel electrophoresis using 10% polyacrylamide slab gels containing 0.1% sodium dodecyl sulfate (SDS) (20). To concentrate the released ^{125}I-HDL, the same chase medium was exposed in succession to four different dishes containing cell-bound ^{125}I-HDL. Fibroblasts grown in 60-mm culture dishes (270 μg protein/dish) were incubated for 2 h at 0°C with medium containing apo E-free ^{125}I-HDL and then thoroughly washed by standard procedures. Serum-free medium containing unlabeled apo E-free HDL (10 μg/ml) was added to the first dish (2.5 ml/dish) and cells were incubated (chase) for 1 h at 37°C. The chase medium was then transferred to the second dish for an additional 1-h incubation at 37°C. This procedure was repeated twice again for the remaining two dishes. The 4°C binding incubations were started at 1-h intervals, so that each dish was exposed to ^{125}I-HDL for the same period of time. After the final chase incubation, the medium was dialyzed against 20 mM NH_4HCO_3 and then lyophilized. The lyophilized sample was dissolved in phosphate/SDS buffer (0.268% $Na_2HPO_4 \cdot 7H_2O$, 0.137% $NaH_2PO_4 \cdot H_2O$, and 1.0% SDS, 10–20% vol/vol glycerol, 0.005% bromophenol blue, at pH 7.0). Electrophoresis was performed at 20–75 mA and 40–140 V for 3–4 h. Small aliquots of the initial unlabeled apo E-free HDL, apo E-free ^{125}I-HDL, and mixtures of known M_r standards were run in parallel to the medium samples. Proteins were visualized by Coomassie blue G-250 staining, and autoradiography was performed by exposure of dried gels to x-ray filter at −70°C for 24–72 h. In some cases, the dried gels were sliced into consecutive 5-mm sections and each section was gamma counted for ^{125}I radioactivity.

Other methods. To estimate the relative rates of sterol synthesis, cells were rapidly washed twice (at room temperature) with PBS and then incubated for 2 h at 37°C with Krebs-Ringer phosphate buffer, pH 7.4, containing 0.5 mM sodium [^{14}C]acetate (30–50 μCi/μmol). To estimate the cellular content of cholesterol oleate, cells were incubated with medium containing [^{14}C]oleic acid bound to albumin prepared as previously described (21). After the incubation times indicated, the dishes were placed on ice, washed twice with ice-cold PBS. Cells were harvested with a teflon policeman into ice-cold CH_3OH:H_2O (2:1), the lipids were extracted with $CHCl_3$, and the amount of label incorporated into total sterol (sterol synthesis) or cholesterol esters was measured as previously described (21). The amount of ^{125}I-LDL degraded by fibroblasts was estimated from the appearance in the medium of TCA-soluble, noniodide radioactivity as previously described (8).

RESULTS

To demonstrate whether the techniques used were yielding an accurate assessment of high-affinity binding of HDL, the release of radioactivity during washing, trypsinization, and cell digestion was monitored after incubation of cultured skin fibroblasts at 37°C with 10 μg/ml ^{125}I-HDL_3 protein (Fig. 1). The HDL_3 subfraction was used because it contains very little apo E. After the fifth wash, essentially no radioactivity was found in the medium. Even when binding was performed with 100 μg/ml ^{125}I-HDL_3, the amount of ^{125}I radioactivity released in the sixth wash was <15% of the final cell-associated radioactivity (data not shown). After the sixth wash, trypsinization of the cells resulted in a significant release of ^{125}I-HDL_3 from the cells (Fig. 1). Washing the cell pellet after trypsinization released very little additional HDL. After digestion of the cell pellet with NaOH, some further radioactivity was recovered. However, the majority of the cell-associated counts (~75%) were released by trypsin; indicating that HDL binding is trypsin sensitive and appears to be confined to the cell surface. To determine whether the amount of cholesterol in the cell had any effect on HDL binding, cells preincubated in 10% whole human serum, which loads the cells with cholesterol, were compared with cells preincubated in 10% LDS, which results in relatively cholesterol-depleted cells. Preincubation with whole human serum did not affect the amount of HDL either loosely associated with the cells (wash-releasable) or tightly bound to the cells (trypsin-releasable).

To minimize internalization of HDL, binding assays were routinely performed at 0°–4°C and cells were digested with NaOH (Methods). Saturation curves for HDL binding to normal fibroblasts were obtained by incubations with increasing concentrations of apo E-free ^{125}I-HDL, prepared from whole HDL that had been subjected to heparin-agarose affinity chromatography. Typically, binding of labeled HDL increased linearly at concentrations > 20 μg HDL protein (Fig. 2). When this linear binding component was subtracted from the total binding, a high-affinity saturable binding component was evident (Fig. 2). A Scatchard (19) plot of the total apo E-free HDL binding yielded a curve indicative of more than one component of binding (Fig. 2, inset). These studies suggest that normal fibroblasts possess low- and high-affinity sites that bind apo E-free HDL.

To estimate the relative amount of nonspecific binding of HDL, normal fibroblasts were incubated with increasing concentrations of apo E-free ^{125}I-HDL plus or minus at least 50-fold excess unlabeled apo E-free HDL. The linear component of HDL binding that occurred in the presence of excess unlabeled HDL

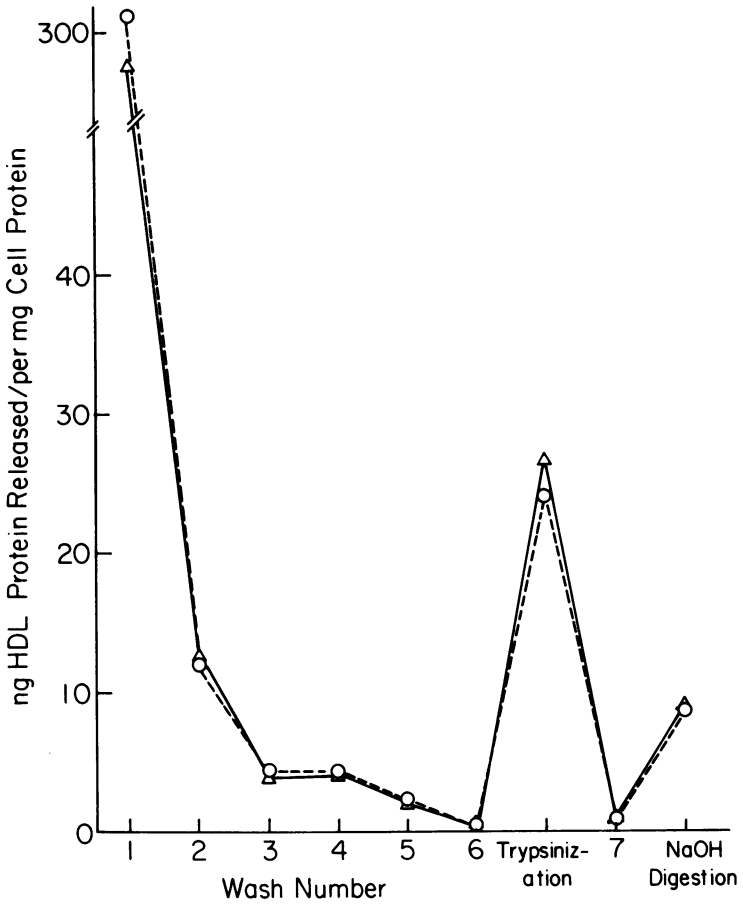

FIGURE 1 Release of surface bound ^{125}I-HDL$_3$ by sequential washes. Normal fibroblasts were preincubated for 30 h in medium containing either 10% whole human serum (△) or 10% LDS (○), followed by an overnight preincubation with serum-free medium plus 2 mg/ml albumin (Methods). The cells were then incubated with 10 μg/ml of ^{125}I-HDL$_3$ protein at 37°C for 4 h. The medium was then removed and the routine washing procedure used in these binding experiments was performed (Methods). The amount of radioactivity released after each wash was determined. After the sixth wash, the cells were trypsinized and the amount of radioactivity released was measured. The cell pellet was then digested with NaOH and amount of radioactivity that had remained associated with the cells was determined.

presumably represented low-affinity, nonspecific binding (Fig. 3). When the nonspecific component was subtracted from total binding, a high-affinity saturable component of binding was apparent. A Scatchard plot of the high-affinity binding was significantly linear ($r = 0.79$, $P < 0.01$) (Fig. 3, inset), indicative of a single class of binding sites.

Another method for determination of binding of HDL to cells independent of the LDL receptor is the incubation of untreated fractions of HDL with cells lacking the LDL receptor. An ultracentrifugally isolated subfraction of HDL that contains very little apo E, HDL$_3$, was iodinated and incubated with LDL receptor-negative fibroblasts (Fig. 4A). Nonspecific binding was determined by calculation of the slope of binding between 100 and 400 μg/ml of ^{125}I-HDL$_3$ protein in the medium and extrapolation back to zero. Subtraction of nonspecific binding from total binding again revealed a high-affinity, saturable component. In a separate experiment, binding of apo E-free ^{125}I-HDL to LDL receptor-negative fibroblasts was also determined (Fig. 4B). Again, a high-affinity, saturable binding component was apparent, which provides further evidence that HDL binds to the cell surface with high affinity, independent of the LDL receptor. Scatchard analysis of the high-affinity binding of ^{125}I-HDL$_3$

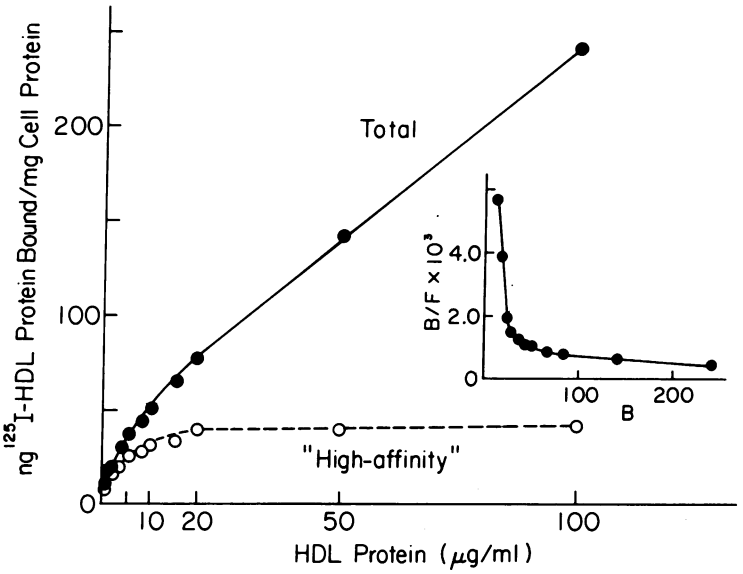

FIGURE 2 Apo E-free HDL binding to normal fibroblasts. Normal fibroblasts were preincubated for 18 h with serum-free medium containing 2 mg/ml albumin and then incubated for 2 h at 0°–4°C with increasing concentrations of apo E-free ^{125}I-HDL. Total binding (●) was measured on NaOH digests of the washed monolayer (Methods). The high-affinity component (○) was calculated by subtraction of values for nonspecific binding at each HDL concentration as estimated from the slope of the linear component of the total binding curve. The inset is a Scatchard plot of total binding where B represents nanograms of HDL protein bound per milligram cell protein and F represents the concentration of HDL in the medium (nanograms of HDL protein per milliliter). The data are from an experiment representative of six different experiments with four different strains of normal fibroblasts.

and apo E-free ^{125}I-HDL yielded linear plots (insets Figs. 4A and B).

Further evidence that HDL binds to cells at a site other than the LDL receptor is the independence of HDL binding from calcium. High-affinity LDL binding was dependent on the presence of calcium in the medium as expected (Table I). In contrast, high-affinity apo E-free HDL binding was the same in the presence or absence of calcium, indicating that HDL binding was independent of calcium. Low-affinity, nonspecific binding of LDL and HDL was unaffected by the availability of calcium in the medium.

Binding of apo E-free ^{125}I-HDL to cultured human arterial smooth muscle cells was also tested (Fig. 5). Again, a high-affinity, saturable component of binding was evident. These binding sites appeared to saturate at ~20 μg/ml HDL protein, similar to the saturation concentration observed for fibroblasts.

To assess specificity of the HDL binding sites, normal fibroblasts were incubated with apo E-free ^{125}I-HDL, plus or minus increasing concentrations of unlabeled lipoprotein subfractions (Fig. 6A). Unlabeled apo E-free HDL competed well for apo E-free ^{125}I-HDL binding as expected. Based on the lipoprotein protein content, the other HDL density subfractions tested (HDL_2, HDL_3, and VHDL, not subjected to affinity chromatography) (Methods) competed as well as apo E-free HDL for binding. In contrast, LDL competed to a much smaller degree. Studies of competitive binding of LDL receptor-negative fibroblasts using untreated HDL_3 (Fig. 6B) indicated that, as with apo E-free HDL binding to normal cells, the various density subfractions of HDL competed well for labeled HDL_3 binding, whereas LDL competed to a much smaller extent. The finding that LDL competes slightly for HDL binding, may be explained by the presence of apo A-I in the LDL density fraction that accounted for ~1.0% of total LDL protein (0.95±0.21%, mean±SD for four preparations). Apparently, the relative sterol content of the HDL particle has little influence on cell surface HDL binding. Preparations of HDL_3 that had been subjected to heptane extraction to remove neutral lipids (Methods) competed nearly as effectively as untreated HDL_3 for binding to fibroblasts (Fig. 7).

It has been noted previously that HDL_3 and VHDL can promote cholesterol efflux from cultured fibroblasts (21). The increase in cholesterol efflux was as-

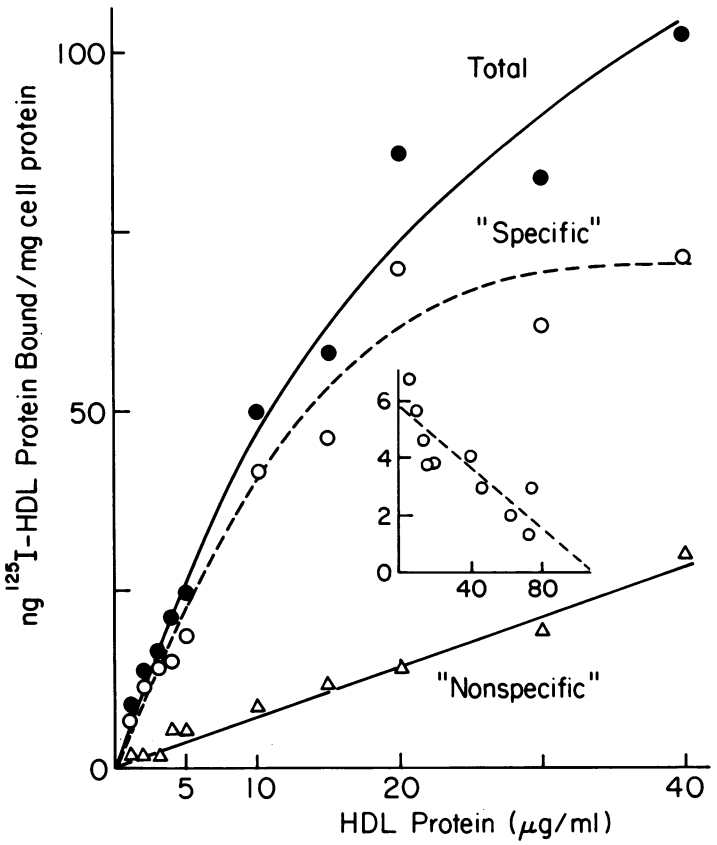

FIGURE 3 The effects of excess unlabeled apo E-free HDL on binding of apo E-free ^{125}I-HDL to normal fibroblasts. Fibroblasts were incubated at 0°C with increasing concentrations of apo E-free ^{125}I-HDL plus (△) or minus (●) at least 50-fold excess unlabeled apo E-free HDL. Binding was measured as described for Fig. 2. Specific binding (○) was calculated by subtraction of the values for binding in the presence of excess unlabeled HDL (nonspecific binding) from the values for total binding. Values for specific binding were plotted by the method of Scatchard (inset) as described in Fig. 2. Linearity of the Scatchard plot was assessed by linear regression analysis ($r = 0.79$, $P < 0.01$).

sociated with increases in LDL receptor activity and the rate of sterol synthesis by the cell. The effects of HDL_3 and VHDL on the apparent rate of cholesterol efflux were concentration dependent, saturating at HDL concentrations that corresponded closely to those observed for saturation of high-affinity HDL binding (20 μg protein/ml). When fibroblasts were incubated for 24 h with increasing concentrations of apo E-free HDL_3, an increase in the rate of sterol synthesis from [^{14}C]acetate occurred that saturated at apo E-free HDL_3 concentrations of 20 μg protein/ml (Fig. 8). Furthermore, the addition of apo E-free HDL_3 to medium containing ^{125}I-LDL increased the cell's ability to degrade LDL during 24-h incubations (Fig. 8). This enhancement of LDL degradation also saturated at ~20 μg/ml HDL protein. The effect of HDL_3 on LDL degradation was apparently due to sustained elevation of LDL receptor activity. Direct measurement of LDL receptor activity also demonstrated that HDL_3 exerted its maximum enhancement at 20 μg protein/ml (Fig. 8C). In contrast, when cells were preincubated with LDS to deplete cells of cholesterol and up-regulate the LDL receptor, HDL_3 had little additional effect on LDL receptor activities (Fig. 8C). Thus, the effect of HDL_3 on cell cholesterol homeostasis became maximal at an HDL_3 concentration similar to that which saturated the high-affinity HDL binding sites on the cell surface.

One explanation for the saturation curves shown in Fig. 8 is that the process of cholesterol efflux becomes rate limiting at the higher concentrations of HDL_3 because of depletion of cellular cholesterol. To test for

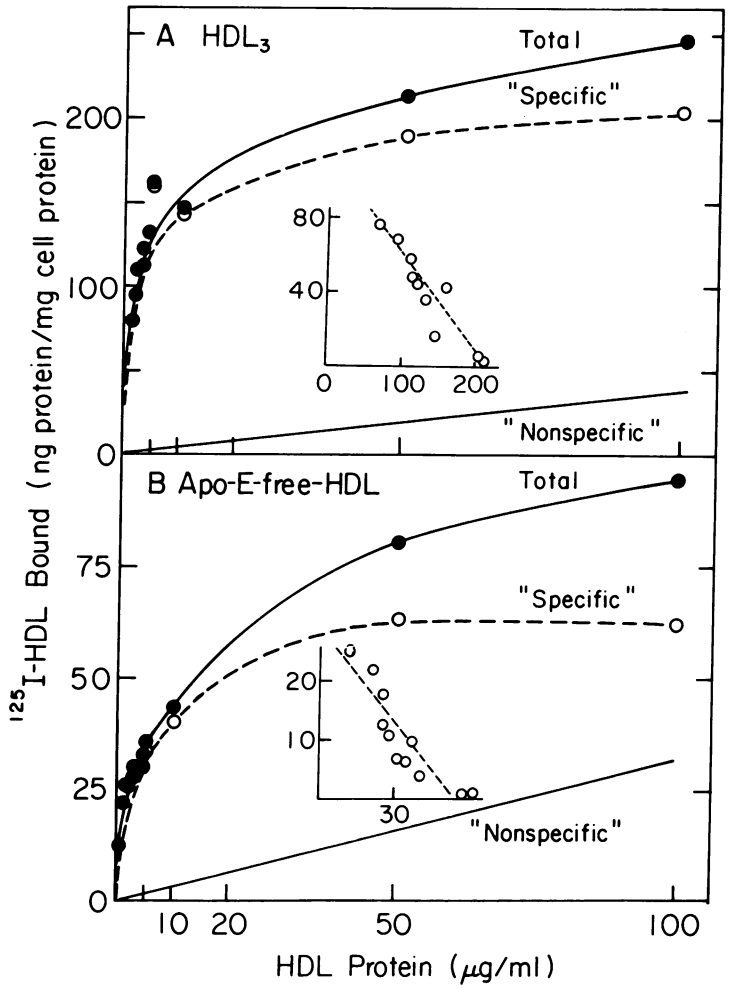

FIGURE 4 Binding of untreated ^{125}I-HDL$_3$ (panel A) and apo E-free ^{125}I-HDL (panel B) to LDL receptor-negative fibroblasts. Binding of HDL was measured as described in Fig. 2 except that receptor-negative fibroblasts (GM2000) were used. The nonspecific component was estimated from the linear slopes of binding between 100 and 400 μg/ml of ^{125}I-HDL$_3$ and apo E-free ^{125}I-HDL protein extrapolated back to zero. Specific binding was estimated by subtraction of nonspecific binding from total binding. Scatchard plots (Fig. 2) for specific binding (insets) were significantly linear (panel A: $r = 0.93$, $P < 0.001$; panel B: $r = 0.83$, $P < 0.001$).

this possibility the relative amount of cholesterol transported from the cell was estimated when fibroblasts were exposed to increasing concentrations of sterol-depleted HDL$_3$ over short- and long-term incubations. Sterol was extracted from HDL$_3$ (Fig. 7) because preliminary results indicated that this procedure increased the ability of HDL$_3$ to promote cholesterol efflux during long-term incubations. The relative cellular cholesterol content was estimated from the incorporation of [^{14}C]oleate into cholesterol esters. Synthesis of cholesteryl [^{14}C]oleate, controlled by the enzymes acyl-CoA cholesterol acyltransferase, is presumed to be a direct function of the "excess" free cholesterol content of the cell (1). During 36-h incubations increasing the medium content of sterol-depleted HDL$_3$ caused a >80% reduction in incorporation of [^{14}C]oleate into cholesteryl ester (Fig. 9), indicating near complete depletion of excess cellular cholesterol even at low (5 μg protein/ml) HDL$_3$ concentrations. In contrast, during 6-h incubations, addition of sterol-depleted HDL$_3$ to the medium caused <30% decrease in cholesteryl [^{14}C]oleate synthesis (Fig. 9). Despite this small change, the effects of sterol-depleted HDL$_3$ saturated at a concentration of 20 μg

TABLE I
Effects of Calcium on Binding of Apo E-free HDL and LDL to Fibroblasts

	^{125}I-Lipoprotein bound	
	+Calcium	−Calcium
	mg/mg cell protein	
Apo E-free ^{125}I-HDL	58.4	60.0
+ excess unlabeled apo E-free HDL	7.91	9.39
^{125}I-LDL	45.5	5.10
+ excess unlabeled HDL	1.64	2.75

Normal fibroblasts were preincubated for 48 h with medium containing 10% LDS and then incubated at 0°–4°C for 2 h with 2 μg/ml of the indicated ^{125}I-lipoprotein plus or minus 200 μg/ml of the same unlabeled lipoprotein in Krebs-Ringer phosphate buffer with (+) or without (−) 1.3 mM calcium. Binding of labeled lipoprotein was determined as described in Methods and in the legend for Fig. 3.

protein/ml, similar to the effects shown in Fig. 8. Apparently, depletion of available cholesterol from the cell is not solely responsible for the saturation of cholesterol efflux observed in the presence of increasing concentrations of HDL_3.

Previous studies have shown that, of the major HDL subfractions, only HDL_3 and denser particles can promote cholesterol efflux from fibroblasts (21). Although increased LDL degradation by fibroblasts occurs in the presence of HDL_3, addition of apo E-free HDL_2 to medium containing ^{125}I-LDL had little effect on LDL degradation (Fig. 10). Furthermore, a mixture of HDL_3 and apo E-free HDL_2 (one-to-one, protein-to-protein) had less ability to enhance LDL degradation than did HDL_3 alone (Fig. 10). Apparently, apo E-free HDL_2 can block the enhancement of LDL degradation promoted by HDL_3.

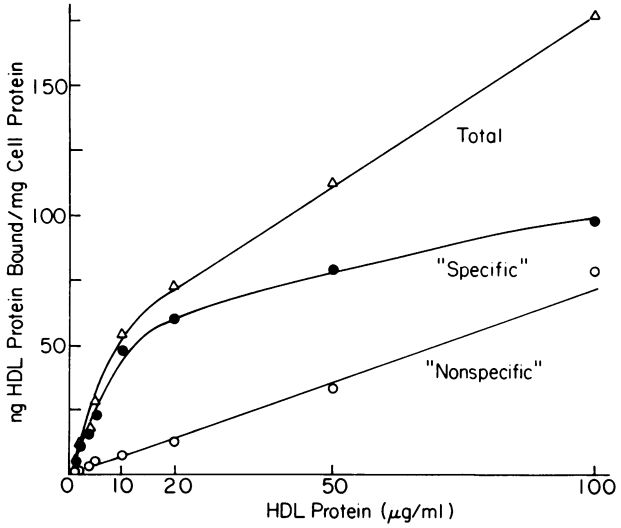

FIGURE 5 Apo E-free ^{125}I-HDL binding to human arterial smooth muscle cells. Total binding was measured as described for Fig. 2. Nonspecific binding was determined from incubations at each concentration of apo E-free ^{125}I-HDL in the presence of 1,000 μg/ml unlabeled apo E-free HDL. Specific binding was calculated by subtraction of nonspecific from total binding.

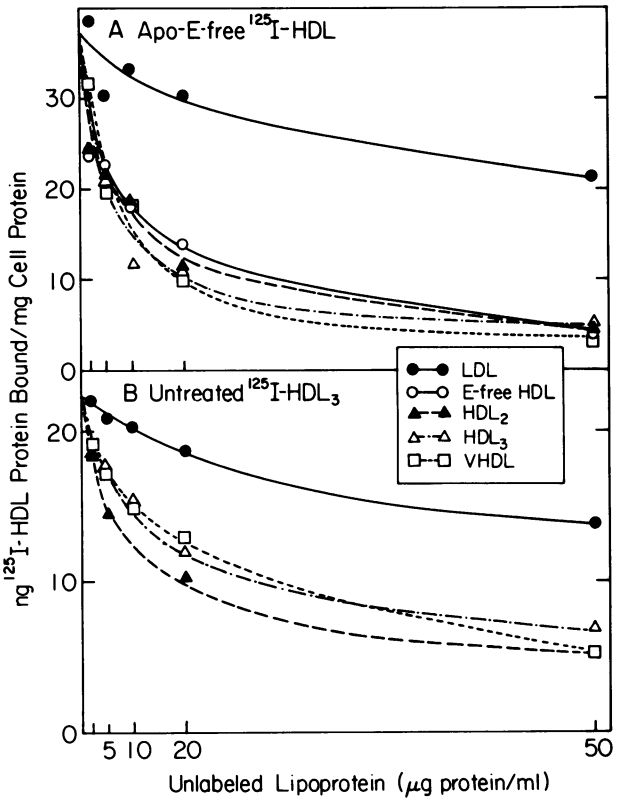

FIGURE 6 Competitive inhibition of apo E-free ^{125}I-HDL binding to normal fibroblasts and ^{125}I-HDL_3 binding to LDL receptor-negative fibroblasts by serum lipoprotein fractions. Normal (panel A) and LDL receptor negative (GM488, panel B) fibroblasts were incubated at 0°C with 2 μg protein/ml apo E-free ^{125}I-HDL and untreated ^{125}I-HDL_3, respectively, in the presence of increasing concentrations of the indicated unlabeled lipoprotein subfractions (Methods). Binding of labeled HDL was measured as described for Fig. 2.

A requirement for the involvement of specific binding for the promotion of cholesterol efflux by HDL is that the bound HDL must be readily displaced from the binding sites so that cholesterol can be transported away from the cell. To test this possibility, fibroblasts were first incubated at 0°C with saturating (20 μg/ml) concentrations of apo E-free ^{125}I-HDL. The washed

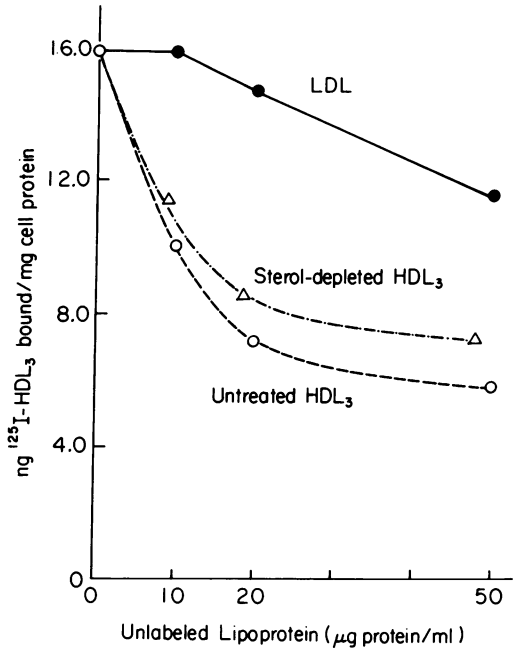

FIGURE 7 Competitive inhibition of ^{125}I-HDL$_3$ binding to fibroblasts by sterol-depleted HDL$_3$. Normal fibroblasts were incubated at 0°C with 5 μg protein/ml ^{125}I-HDL$_3$ in the presence of increasing concentrations of the indicated unlabeled lipoprotein subfractions. Sterol was extracted from HDL$_3$ with heptane as described under Methods. Binding of ^{125}I-HDL$_3$ was measured as described for Fig. 2.

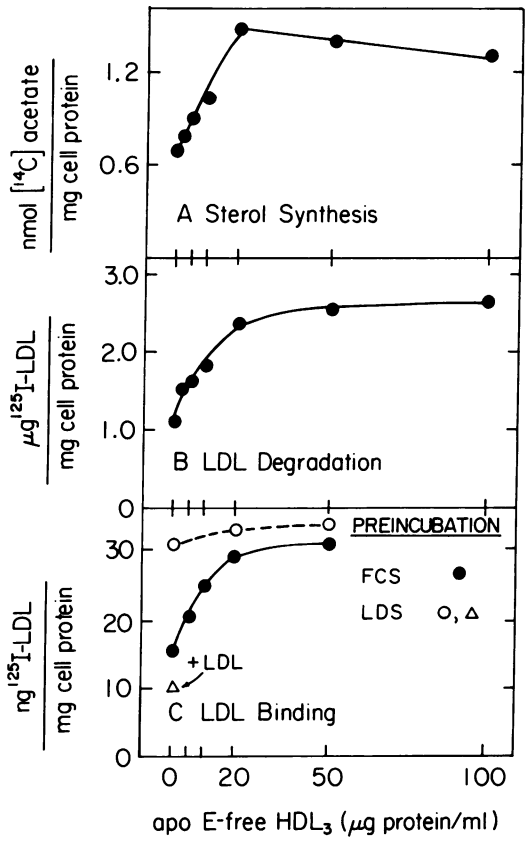

FIGURE 8 Effect of apo E-free HDL$_3$ on sterol synthesis and ^{125}I-LDL degradation and binding by fibroblasts. For panel A, fibroblasts were preincubated for 4 h in a serum-free albumin medium before the addition of fresh serum-free albumin medium containing the indicated concentration of apo E-free HDL$_3$. After 24 h, cells were washed, pulse incubated for 2 h with [^{14}C]acetate, and harvested for lipid extractions (Methods). For panel B, cultured fibroblasts were preincubated for 18 h in serum-free medium containing 2 mg/ml albumin before the addition of fresh serum-free medium containing 2 mg/ml albumin, 30 μg/ml ^{125}I-LDL protein and the indicated concentration of apo E-free HDL$_3$. After 24 h, the amount of ^{125}I-LDL degraded was measured as described in Methods. For panel C, cultured fibroblasts were preincubated in the presence of either 10% fetal calf serum (●) or 10% human LDS (○, △). After 48 h, cells were incubated with serum-free medium containing 2% albumin plus the indicated concentration of HDL$_3$. Duplicate dishes were incubated with medium containing 10 μg/ml LDL protein (△). After 24 h, the cells were washed twice with PBS at room temperature to allow for displacement of prebound lipoproteins and then chilled to 0°C while bathed with the third wash medium. ^{125}I-LDL binding was measured as described under Methods. The results for each panel are from separate experiments using different cell strains and different HDL$_3$ preparations.

monolayers were then warmed to 37°C and the fate of the prebound HDL was monitored (Fig. 11A and B). With serum- and lipoprotein-free medium, nearly 40% of the bound (trypsin releasable) apo E-free ^{125}I-HDL was displaced during the first 2 h of incubation. This displacement was increased to >60% when unlabeled apo E-free HDL was added to the medium. Nearly all of the displaced ^{125}I radioactivity was recovered in the medium as TCA-insoluble derivatives, suggesting that release of intact HDL particles from the surface accounted for the displacement of prebound HDL. Virtually none of the apo E-free ^{125}I-HDL was internalized or degraded by the cell. In contrast, prebound apo E-free ^{125}I-HDL was not displaced if cells were maintained at 0°C during the 4-h incubations (Fig. 10C and D). The only ^{125}I-HDL released at 0°C appeared to be a relatively small pool of trypsin-resistant radioactivity that was insoluble in TCA. This release at 0°C was unaffected by the presence of unlabeled apo E-free HDL. These results indicate that binding of HDL to the cell surface is reversible and that the displacement of prebound HDL is temperature dependent.

To characterize the HDL particles that were bound to the fibroblasts at 0°C and subsequently released into medium at 37°C, the released ^{125}I-HDL was subjected to SDS-polyacrylamide gel electrophoresis (Methods).

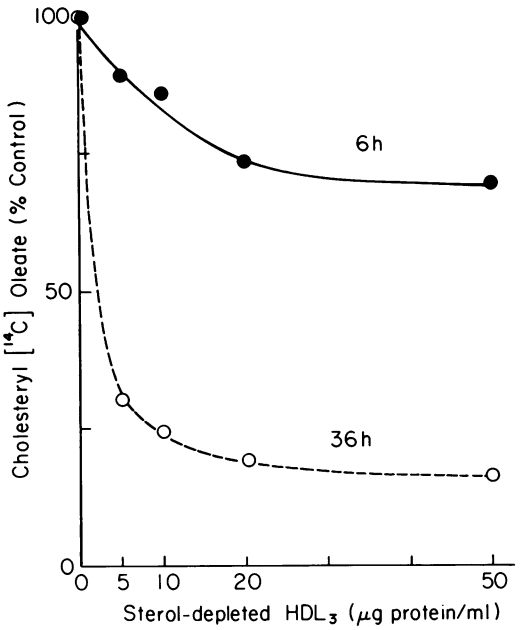

FIGURE 9 Effects of sterol-depleted HDL_3 on cholesteryl [^{14}C]oleate synthesis by fibroblasts. To load cells with cholesterol, fibroblasts were preincubated for 48 h with 10% human LDS to up-regulate the LDL receptor followed by 24-h incubation with medium containing 10% LDS plus 50 μg protein/ml LDL. After 24 h, cells were washed twice with PBS containing albumin and then incubated with medium containing [^{14}C]oleic acid bound to albumin plus the indicated concentration of sterol-depleted HDL_3, prepared as described in Methods. After the indicated times, the dishes were chilled to 0°C, and cell lipids were extracted for measurement of cholesteryl [^{14}C]oleate content (Methods). The medium concentrations of [^{14}C]oleic acid/albumin for 6- and 36-h incubations were 72 μM/0.2 and 140 μM/0.5%, respectively. Each value represents the mean of two to three dishes expressed as percent values for lipoprotein-free dishes (control). Mean values (±SEM, $n = 3$) for controls were 9.6±0.3 and 11.0±0.1 nmol cholesteryl [^{14}C]oleate/mg cell protein for 6- and 36-h incubations, respectively.

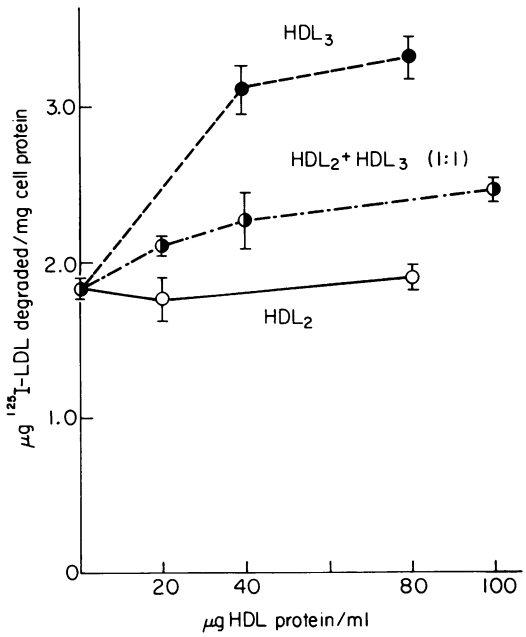

FIGURE 10 Effects of subfractions of apo E-free HDL on degradation of ^{125}I-LDL by cultured fibroblasts. Cultured fibroblasts were preincubated with 10% fetal calf serum, washed twice with PBS, and then incubated with serum-free medium containing 2 mg/ml albumin, 20 μg/ml ^{125}I-LDL and increasing concentrations of apo E-free HDL_2 (d = 1.063–1.125), HDL_3 or a one-to-one (protein-to-protein) mixture of HDL_2 plus HDL_3. Whole HDL (d = 1.063–1.21) was first subjected to heparin-agarose affinity chromatography to remove apo E and B before separation into HDL_2 and HDL_3 by ultracentrifugation. Each value represents the mean±SEM of three incubations.

Coomassie blue staining of electrophoretic gels of the original unlabeled apo E-free HDL preparation revealed two major bands corresponding to apo A-I and A-II (Fig. 12). Iodination of the HDL preparation yielded particles that were predominantly labeled in apo A-I and A-II as evident from autoradiograms of the ^{125}I-HDL gels (Fig. 12). As determined on consecutive 5-mm slices of ^{125}I-HDL gels, ~60 and 30% of the radioactivity was associated with apo A-I and apo A-II, respectively, with no other radioactive peaks evident (data not shown). When the ^{125}I-HDL was immunoprecipitated (22), 92 and 75% of the radioactivity was precipitated by antibodies to apo A-I and A-II, respectively. These results suggest that most of the ^{125}I-HDL particles contained both apo A-I and A-II, but a small proportion of the apo A-I-containing particles were devoid of apo A-II (22). When fibroblasts were incubated with medium containing ^{125}I-HDL at 0°C, thoroughly washed, and subsequently chase-incubated at 37°C with medium containing unlabeled HDL (Methods), the radioactive material released from the cell surface had electrophoretic properties that were identical to the unincubated ^{125}I-HDL (Fig. 12). These results strongly suggest that fibroblasts bind and subsequently release the apo A-I- and A-II-containing HDL particles without marked alteration of their protein composition.

DISCUSSION

Recent studies have demonstrated that HDL can bind to high-affinity sites on cells from a variety of different tissues, including rat adrenal gland (2, 3), rat testes (4), rat and canine liver (23–26), bovine endothelium (25), and human skin fibroblasts (6, 28, 29). The physiological significance of this HDL binding has yet to be elucidated. Binding of HDL to steroid hormone-

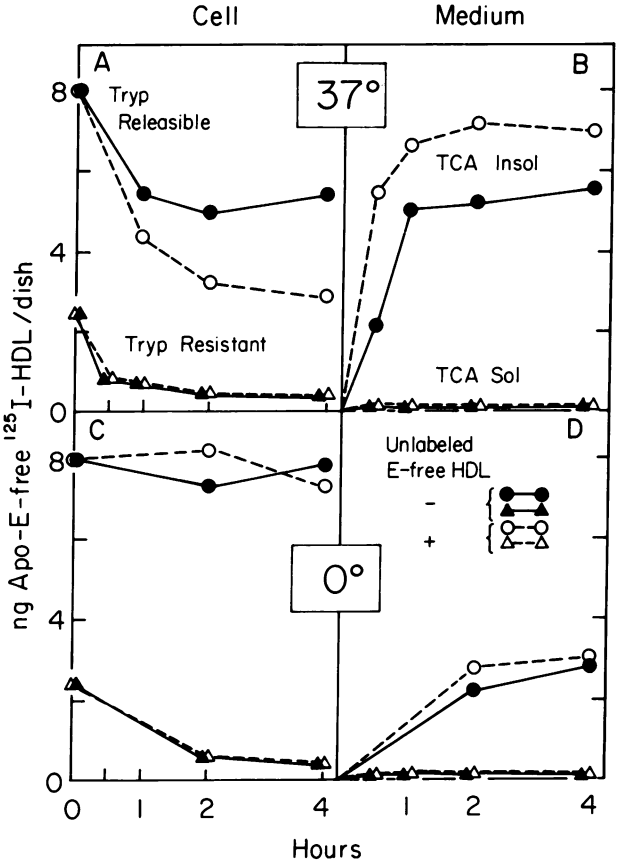

FIGURE 11 The fate of apo E-free ^{125}I-HDL bound to fibroblasts at 0°C after subsequent incubations at 0° or 37°C. Fibroblasts were incubated for 2 h at 0°C with 20 μg protein/ml apo E-free ^{125}I-HDL and thoroughly washed, as described in Methods for the HDL binding assay. Cold serum-free medium was added to each dish that contained 2 mg/ml albumin alone (●, ▲) or albumin plus 20 μg protein/ml unlabeled apo E-free HDL (○, △). One set of dishes was then warmed to 37°C (panels A and B), while the other set was maintained at 0°C (panels C and D). After the times indicated, the medium was collected (panels B and D), treated with TCA, and TCA-soluble (▲, △) and -insoluble (●, ○) radioactivity was measured (Methods). The washed monolayers were then trypsinized (panels A and C), and trypsin-releasable (●, ○) and trypsin-resistant (▲, △) radioactivity was measured (Methods).

producing tissues may act to supply cholesterol to the cell for steroid synthesis (2–4). On the other hand, HDL binding to peripheral cells may promote cholesterol efflux for transport to the liver (5, 6, 21). At present, however, there is no direct experimental evidence showing that cell surface binding of HDL is required for net transport of cholesterol between the cell and HDL particles. It was the purpose of the present study to identify and characterize HDL binding sites on cultured human skin fibroblasts and human arterial smooth muscle cells and to test the hypothesis that modulation of cell cholesterol homeostasis by HDL involves binding of HDL to specific high-affinity sites on the cell surface.

HDL is a heterogenous lipoprotein fraction containing several different subpopulations of particles. Of these subpopulations, those containing apo E can bind avidly to the LDL receptor (7), which may have accounted for much of the observed cell-surface binding of whole HDL reported in some previous studies. To evaluate HDL binding to sites distinct from the LDL receptor, HDL was first subjected to heparin-agarose affinity chromatography to remove apo E- and apo B-containing particles (13). When tested on normal fibroblasts, these apo E-free HDL preparations did not compete at all for LDL binding to the cell. As evident from autoradiograms of SDS electrophoresis gels, radiolabeling of apo E-free HDL with ^{125}I yielded particles with >90% of the radioactivity associated with apo A-I and A-II. Cultured cells were then incubated with increasing concentrations of apo E-free ^{125}I-HDL with and without excess unlabeled apo E-free HDL. Saturation curves of the data from studies with cultured fibroblasts revealed two components of HDL binding. One component, evident as a straight line at apo E-free HDL concentrations >20 μg protein/ml, presumably represented nonspecific binding. The second component appeared to saturate at apo E-free HDL concentrations of ~20 μg protein/ml, suggesting the presence of specific high-affinity binding sites. Curvilinear Scatchard plots of the total binding of labeled apo E-free HDL to fibroblasts supported the existence of high-affinity and low-affinity binding sites. After subtraction of the nonspecific binding, the Scatchard plots were significantly linear, suggestive of one class of high-affinity binding sites. In addition to human fibroblasts, cultured human arterial smooth muscle cells appear to have high-affinity binding sites for apo E-free HDL that also saturate at ~20 μg protein/ml.

Several different lines of evidence indicate that the binding sites for apo E-free HDL are distinct from the cell surface receptor that bind LDL. First, high-affinity binding of apo E-free HDL to normal fibroblasts is calcium independent. In contrast, high-affinity LDL binding to fibroblasts is completely dependent on calcium (30). Moreover, exposure of fibroblasts to pronase or concanavalin A had no effect on HDL binding (data not shown), whereas both agents alter binding of LDL to fibroblasts (30, 31). Pronase-insensitive HDL binding to fibroblasts has also been described by Wu et al. (6) and Miller et al. (28). Cultured fibroblasts from subjects with homozygous familial hypercholesterolemia, which lack LDL receptors (1), also appear to have high-affinity binding sites for apo E-free HDL

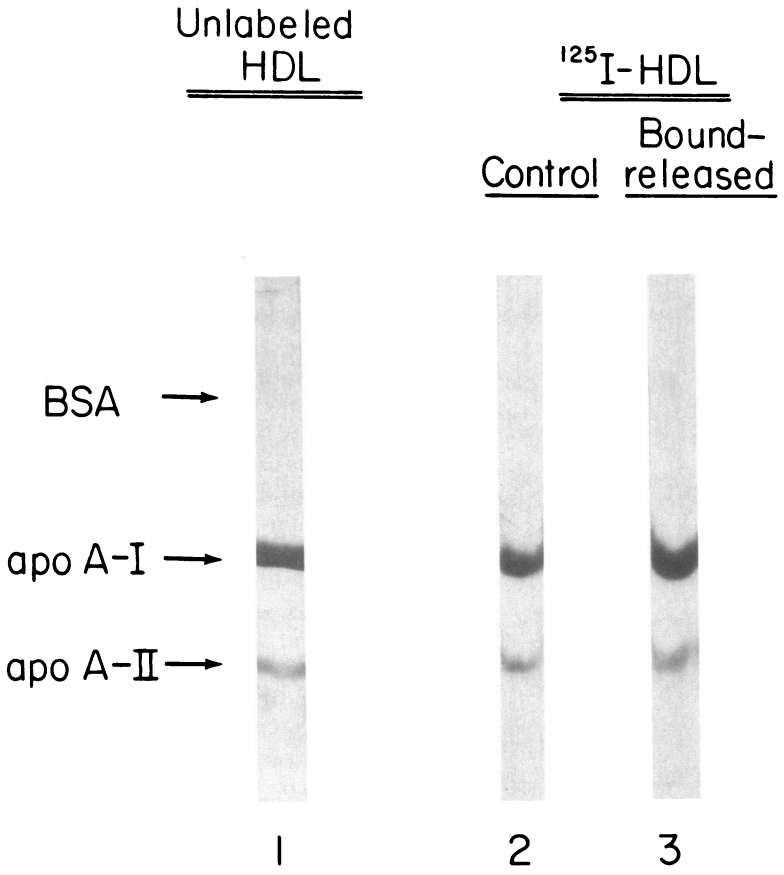

FIGURE 12 Electrophoresis of apo E-free ^{125}I-HDL bound and dissociated from fibroblasts. Fibroblasts were incubated for 2 h at 0°C with 20 μg protein/ml apo E-free ^{125}I-HDL, thoroughly washed, and subsequently chase-incubated for 1 h at 37°C with serum-free medium containing 10 μg protein/ml unlabeled apo E-free HDL (Methods). The chase medium was dialyzed, lyophilized, and subjected to SDS/polyacrylamide slab gel electrophoresis and autoradiography (lane 3), as described in Methods. For control values (lane 2), the unincubated apo E-free ^{125}I-HDL-binding medium was diluted with unincubated chase medium to approximate the number of counts per minute released from the cell (2,000 cpm), and then subjected to the same procedure as the incubated chase medium. Lane 1 represents Coomassie blue stain of the original unlabeled apo E-free HDL preparation.

that saturate at HDL concentrations of ~20 μg protein/ml.

Additional evidence that HDL binding is distinct from the LDL receptor was provided by competitive binding studies. Incubations with increasing concentrations of unlabeled HDL could almost completely block binding of apo E-free ^{125}I-HDL to the cell surface, while LDL at a concentration ratio of 50:1 (LDL to HDL protein) could decrease HDL binding by <40%. The competitive binding studies also demonstrated that the heparin-agarose affinity chromatography treatment itself did not markedly alter the ability of the HDL particle to bind to the cell surface. Based on lipoprotein protein content, untreated subfractions of HDL (i.e., HDL_2, HDL_3, and VHDL; Methods) appeared to compete as effectively as unlabeled apo E-free HDL for binding of iodinated apo E-free HDL. Furthermore, the saturation curves for binding of apo E-free ^{125}I-HDL and untreated ^{125}I-HDL_3 to LDL receptor-negative fibroblasts had similar profiles.

Precise kinetic data for HDL binding was difficult to obtain because of a high degree of variability from one experiment to another. Although saturation of high-affinity binding occurred at similar HDL concentrations, the maximum amount of HDL bound to the cell varied as much as fivefold between experiments. The reason for this variability is presently unknown; however, it may be related to regulation of HDL binding by some as yet unidentified experimen-

tal conditions. Up-regulation of HDL binding to bovine endothelial cells by 25-hydroxycholesterol has been reported by Tauber et al. (27). The possibility that HDL binding by fibroblasts can be regulated is currently under investigation in our laboratory.

Competitive binding studies indicated that, on a total HDL protein basis, unlabeled HDL_2, HDL_3, VHDL, and apo E-free HDL competed equally well for binding of apo E-free ^{125}I-HDL to normal fibroblasts; and HDL_2, HDL_3, and VHDL competed to a similar extent for binding of ^{125}I-HDL_3 to receptor-negative fibroblasts. Based on the HDL apo A-I content, which constitutes 60–80% of the total protein, each HDL subfraction also appeared to compete to a similar extent for the same binding sites. However, competition curves for each HDL subfraction were markedly different when the results were plotted according to the apo A-II content (data not shown). Assuming that the binding ligand is a protein, these competitive binding studies suggest that apo A-I, rather than apo A-II, is the major ligand that binds to the high-affinity sites. The lipid composition of the different HDL subfractions appears to have little influence on their ability to compete for cell surface binding. Both the sterol and phospholipid to total protein ratios vary severalfold between subfractions. Moreover, extraction of sterol from HDL_3 by heptane had little effect on its ability to compete for ^{125}I-HDL_3 binding to fibroblasts.

Binding of apo E-free HDL to the cell surface is readily reversible. When fibroblasts were warmed to 37°C after apo E-free ^{125}I-HDL was bound to the cell surface at 0°C, most of the surface bound radioactivity was displaced within 2 h in the presence of unlabeled apo E-free HDL. All of the radioactivity appeared as TCA-insoluble derivatives in the medium, while virtually none of the bound HDL was internalized or degraded by the cell. Even in the absence of unlabeled apo E-free HDL, a marked portion of the prebound apo E-free ^{125}I-HDL was released from the cell surface. Based on comparison of autoradiograms of electrophoresis gels, the released ^{125}I-HDL had the same properties as the original ^{125}I-HDL and thus appeared to be unaltered in the process. In contrast, when cells were maintained at 0°C, the prebound HDL was not released from the cell surface regardless of the presence or absence of unlabeled apo E-free HDL. Reversibility of HDL binding supports the proposal that the role of HDL is to transfer cholesterol directly from extrahepatic cells rather than deliver cholesterol by an endocytotic process characteristic of the LDL receptor pathway (1).

Recent studies from several laboratories have shown that HDL_3 and/or denser apo A-I-containing particles can promote cholesterol efflux from cultured human fibroblasts (21, 32–34), monkey arterial smooth muscle cells (35), and mouse peritoneal macrophages (36). Presumably in response to increased cholesterol efflux, exposure of cells to HDL_3 increases the activity of the LDL receptor (21, 32), enhances the rate of LDL degradation (37), increases the rate of sterol synthesis (21, 34, 38), and decreases incorporation of oleate into cell cholesteryl esters (21, 36). Results from this study indicate that apo E-free HDL_3 can enhance the ability of fibroblasts to bind and degrade ^{125}I-LDL and to synthesize sterol from [^{14}C]acetate. The effects of apo E-free HDL_3 on these processes became maximal at a concentration of 20 μg protein/ml, the same concentration that appeared to saturate the high-affinity sites that bind apo E-free HDL. Studies by Daniels et al. (33), using sterol-depleted HDL, also have shown that the rate of cholesterol efflux from cultured fibroblasts saturates at 20 μg HDL protein/ml. These results conform to the proposal that cholesterol efflux is mediated by specific high-affinity binding of HDL particles to the cell surface. Apparently, depletion of cellular cholesterol or saturation of the rate of cholesterol transport per se could not account for the saturating effects of HDL_3. First, saturation of cholesterol efflux was evident in the presence of increasing concentrations of sterol-depleted HDL_3 when the incubation time was short enough to allow for only partial depletion of available cellular cholesterol. Second, at concentrations that evoke maximum responses, both sterol-depleted HDL_3 and VHDL (21, 37) promote cholesterol efflux to a greater extent than HDL_3. Thus, the maximum amount of cholesterol that can be transported from the cell depends on both the medium concentration and physical properties of the HDL particles.

In contrast to HDL_3 and denser apo-AI-containing particles, HDL_2, cannot promote cholesterol efflux from cultured fibroblasts (21, 38). This is probably because HDL_2 is richer in sterol than the denser subfractions and, thus, does not establish a sufficient diffusion gradient for net transport of cholesterol from cell membranes. If cholesterol efflux is mediated by binding of HDL_3 to a finite number of sites on the cell surface, and if both HDL_3 and HDL_2 bind to these sites, then HDL_2 should have the ability to block the cholesterol efflux promoted by HDL_3. This proposal is supported by results showing that a mixture of apo E-free HDL_2 and HDL_3 had less ability to enhance degradation of ^{125}I-LDL by fibroblasts than did HDL_3 alone. Other studies have demonstrated that HDL_2 can block the stimulatory effects that both HDL_3 and VHDL have on LDL receptor activity over both short- (4 h) and long-term (48 h) incubations (37). Although mechanisms other than competition for the same binding sites may explain the blocking effects of HDL_2, these results emphasize that the major determinant of

the rate of cholesterol efflux from the cell may be the relative proportion of HDL subfractions in the extracellular environment rather than the absolute concentration of HDL particles. This hypothesis would have particular significance in vivo, where the total HDL concentration in extravascular fluids and serum is likely to be 5 to 50 times higher (39) than the concentration required to promote maximum cholesterol efflux from the cell and saturate the high-affinity HDL binding sites on the cell surface. The interaction of HDL particles with the cell surface of extrahepatic cells and the resulting effects on cell cholesterol metabolism may be related to the strong inverse correlation between serum HDL cholesterol levels and the risk for development of atherosclerosis.

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