Regulation of High Density Lipoprotein Receptor Activity in Cultured Human Skin Fibroblasts and Human Arterial Smooth Muscle Cells

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ABSTRACT Cultured human skin fibroblasts and human arterial smooth muscle cells possess high-affinity binding sites specific for high density lipoproteins (HDL). Results from the present study demonstrate that binding of HDL to these sites is up-regulated in response to cholesterol loading of cells. When fibroblasts or smooth muscle cells were preincubated with nonlipoprotein cholesterol, cellular binding of ^{125}I-HDL_3 was enhanced severalfold. This enhancement was sustained in the presence of cholesterol but was readily reversed when cells were exposed to cholesterol-free medium. The stimulatory effect of cholesterol treatment was prevented by cycloheximide, suggesting the involvement of protein synthesis. Kinetic analysis of HDL_3 binding showed that prior exposure to cholesterol led to an induction of high-affinity binding sites on the cell surface. In the up-regulated state, the apparent dissociation constant (K_d) of these sites was ~2 μg protein/ml. Competition studies indicated that the HDL binding sites recognized either HDL_3 or HDL_2 but interacted weakly with low density lipoprotein (LDL). Exposure of cells to lipoprotein cholesterol in the form of LDL also enhanced HDL binding by a process related to delivery of sterol into cells via the LDL receptor pathway. Enhancement of HDL binding to fibroblasts by either nonlipoprotein cholesterol or LDL was associated with an increased cell cholesterol content, a suppressed rate of cholesterol synthesis, decreased LDL receptor activity, and an enhanced rate of cholesterol ester formation. A comparison of HDL_3 binding with the effects of HDL_3 on cholesterol transport from cells revealed similar saturation profiles, implying a link between the two processes. Thus, cultured human fibroblasts and human arterial smooth muscle cells appear to possess specific receptors for HDL that may function to facilitate cholesterol removal from cells.

INTRODUCTION

Studies from several laboratories have shown that human high density lipoproteins (HDL) can promote cholesterol efflux from a variety of different types of cultured extrahepatic cells, including fibroblasts (1–5), arterial smooth muscle cells (5, 6), and mouse peritoneal macrophages (7, 8). These results conform to the hypothesis that HDL serves to transport cholesterol from extrahepatic cells to the liver for ultimate excretion in the bile (9). The first step in this pathway of "reverse cholesterol transport" may be the interaction of HDL particles with the cell membrane, a process that could modulate the rate of flux of cholesterol through the entire pathway. Recent studies from our laboratory have demonstrated that human fibroblasts and human arterial smooth muscle cells possess high-affinity HDL binding sites distinct from the apolipoprotein B, E (apo B, E)[1] receptor (10). The saturation curves for high-affinity HDL binding and for the effects of HDL on cholesterol efflux from cells were strikingly similar, in that both processes appeared to saturate at an HDL protein concentration of 20 μg/ml (1, 3, 10). These results provided evidence that promotion of cholesterol efflux from cells by HDL may be mediated by binding to high-affinity sites on the cell surface. If these binding sites represent true receptors, which function to facilitate removal of cholesterol from cells, then it is possible that cellular binding of HDL is regulated in parallel to changes in the cell cholesterol content. It was the purpose of this study

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[1] *Abbreviations used in this paper:* apo A-I, A-II, B, and E, apolipoprotein A-I, A-II, B, and E; LDS, lipoprotein-deficient serum.

to test this possibility. Results suggest that binding of ^{125}I-HDL$_3$ to cultured human fibroblasts and human arterial smooth muscle cells is up-regulated in response to intracellular accumulation of cholesterol.

METHODS

Cells. Normal human skin fibroblasts were grown from explants of punch biopsies of skin from the inner thighs of normal volunteers. Human arterial smooth muscle cells were grown from explants of intimal-medial segments of thoracic aorta obtained during vascular surgery by methods described previously (11). Cells were grown in plastic tissue culture flasks in modified Dulbecco-Vogt medium containing 10% fetal bovine serum (growth medium) at 37°C, in humidified incubators equilibrated with 5% CO_2, 95% air. Cells were trypsinized from stock flasks (two to eight passages) and seeded in 35-mm plastic petri dishes using 2 ml of medium containing ~5×10^4 cells. Cells were grown in growth medium and used just before reaching confluency, usually 6–10 d after plating. Cells were then washed twice with a phosphate-buffered saline (PBS)-albumin medium (0.2 g/liter KCl, 0.2 g/liter KH_2PO_4, 8.0 g/liter NaCl, 2.16 g/liter $Na_2HPO_4 \cdot 7H_2O$, 2.0 g/liter bovine serum albumin, at pH 7.4) and incubated with serum-free culture medium containing 2.0 g/liter albumin plus the indicated sterol derivatives. Nonlipoprotein cholesterol was dissolved in 95% ethanol (10 mg/ml) before addition to the albumin medium. The final ethanol concentration in the incubation medium never exceeded 1%, equivalent to a final concentration of cholesterol of 100 μg/ml. After the preincubation periods, the indicated parameters of cell cholesterol metabolism were assayed. 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 (12). 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 = 1.063–1.125); and high density$_3$ (HDL$_3$, d = 1.125–1.21). LDL and HDL$_3$ were iodinated with ^{125}I by the McFarlane monochloride procedure as modified for lipoprotein (13). Typically, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10), this method yielded ^{125}I-HDL$_3$ preparations with specific activities of 1×10^8 cpm/mg protein and with >80% of the radiolabel associated with apo A-I and A-II. The major secondary component in the HDL$_3$ preparations that was iodinated was albumin. To minimize interactions of radiolabeled albumin with cells, the binding medium routinely contained excess amounts of unlabeled albumin. As determined by $CHCl_3/CH_3OH$ (2:1, vol/vol) treatment, <10% of radiolabel was associated with lipid. 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. Fractions of HDL that were devoid of apo B and E were prepared by heparin-agarose affinity column chromatography as described previously (10, 14).

Binding of ^{125}I-HDL$_3$ to cells. To determine cell binding of ^{125}I-HDL$_3$ at 37°C, cells were rapidly washed three times with PBS-albumin medium (wash medium) and incubated at 37°C with serum-free culture medium containing 2.0 mg/ml albumin and the indicated concentrations of ^{125}I-HDL$_3$. After the times indicated, usually 1 h, dishes were chilled on ice, washed rapidly three times with ice-cold wash medium, incubated twice for 10 min with the same medium, and washed twice again rapidly with cold wash medium containing no albumin. Cells were then digested in 0.1 N NaOH and an aliquot was assayed for ^{125}I radioactivity and another aliquot was assayed for protein content (15). When the binding assay was performed at 0°C, cells were washed twice at room temperature and chilled to 0°C while being bathed with the third wash medium. Cells were then incubated at 0°C with serum-free medium containing 2.0 mg/ml albumin, 10 mM Hepes (pH 7.4), and the indicated amount of ^{125}I-HDL$_3$. After 2 h, cells were washed seven times by the same procedure used for the 37°C binding assay and digested with NaOH. In some cases, surface-bound ^{125}I-HDL$_3$ was released by treating the washed cells for 10 min at 37°C with medium containing 0.05% trypsin plus EDTA. Cells were then chilled on ice and pelletted by centrifugation at 4°C. Radioactivity in supernatant and cell pellet was assayed to quantify trypsin-releasable ("bound") and trypsin-resistant ("internalized") ^{125}I-HDL$_3$, respectively (10, 11). Previous studies (10) demonstrated that the major components in the ^{125}I-HDL fraction that bound to cells were the apo A-I- and A-II-containing particles.

Sterol synthesis, cholesterol esterification, [^{3}H]cholesterol efflux, and sterol mass. To measure the rate of incorporation of oleic acid into cellular sterol, cells were washed three times with PBS and incubated at 37°C with serum-free culture medium containing [^{14}C]oleic acid (20–30 μM, 2 μCi/ml) bound to albumin (0.05–0.10%). After 1 h, the dishes were chilled on ice and washed twice with ice-cold PBS. Cellular lipids were extracted by the hexane-isopropanol method described by Brown et al. (7). The extracted lipids were taken to dryness under N_2 at 60°C, resolubilized in a small amount of $CHCl_3$, and separated by thin-layer chromatography, by methods previously described (16). The esterified and unesterified sterol silica spots were scraped into glass-stoppered tubes and the lipids were saponified at 70°–80°C for 1 h with 1.0 ml 1 N KOH in 95% ethanol. To separate free cholesterol from the fatty acyl moieties, 1.5 ml H_2O and 4.0 ml hexane were added, the tubes were vigorously shaken, and the phases were separated by mild centrifugation. To measure cholesteryl [^{14}C]oleate, an aliquot of the water-ethanol phase of saponified cholesteryl ester was counted. To measure [^{14}C]cholesterol, an aliquot of the hexane phase of saponified unesterified cholesterol was counted. Preliminary studies demonstrated that use of [^{14}C]oleate and [^{14}C]acetate to assess relative rates of sterol synthesis by fibroblasts gave comparable results, suggesting that the rate of oxidation of oleate by cells was fast enough to provide adequate acetyl units for cholesterol synthesis. Use of [^{14}C]oleate, however, tended to lead to higher silica-plate background counts than did use of [^{14}C]acetate. Since it was possible to measure relative rates of cholesteryl esterification and sterol synthesis on the same dishes, [^{14}C]oleate was routinely used in the current studies. Esterified and unesterified cholesterol mass was measured in aliquots of the hexane phase of the saponified silica spots by the method of Heider and Boyett (17). When cells were preincubated with [^{3}H]cholesterol, the amount of radiolabel incorporated into cell sterols was determined by counting aliquots of the same hexane phases used to determine cholesterol mass. To assay the rate of efflux of cholesterol from cells, fibroblasts prelabeled with [^{3}H]cholesterol were subsequently incubated for 4 h with nonradioactive medium, and the amount of ^{3}H-radioactivity appearing in the medium was measured.

Other methods. Binding of ^{125}I-LDL to fibroblasts was determined by the same procedure described for the 0°C assay of ^{125}I-HDL$_3$ binding, except that ^{125}I-LDL was released

from washed cell monolayers by dextran sulfate treatment as described previously (16). For studies in which the fate of prebound ^{125}I-HDL$_3$ was assessed, the chase medium was treated with trichloroacetic acid (TCA) (10% final concentration) to precipitate proteins. The ^{125}I-radioactivity in the TCA precipitate and supernatant were assumed to represent dissociation of intact ^{125}I-HDL$_3$ from the cell surface and degradation of ^{125}I-HDL$_3$ by cells, respectively (10).

RESULTS

Initial studies were conducted to examine the effect of nonlipoprotein cholesterol on HDL binding to cultured human fibroblasts and human arterial smooth muscle cells, since cholesterol in this form enters cells by a receptor-independent process. When cells were preincubated with serum-free medium containing albumin plus nonlipoprotein cholesterol followed by 1-h pulse incubation with ^{125}I-HDL$_3$, binding of HDL$_3$ to both fibroblasts and smooth muscle cells increased with increasing concentrations of cholesterol (Fig. 1).

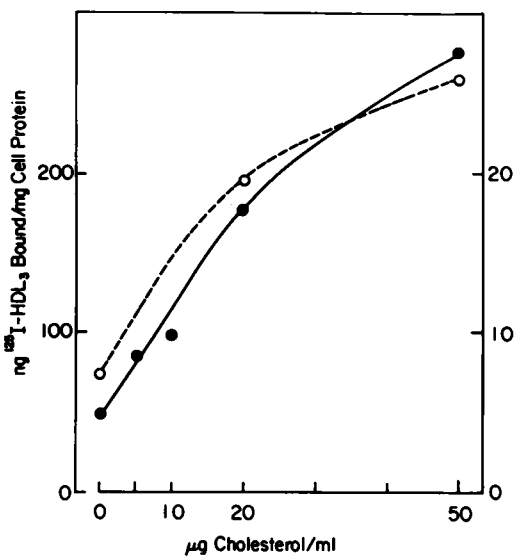

FIGURE 1 Effect of nonlipoprotein cholesterol on ^{125}I-HDL$_3$ binding to cultured human skin fibroblasts and human arterial smooth muscle cells. Cultured fibroblasts (●) and arterial smooth muscle cells (○) were incubated with serum-free medium containing 0.2% albumin and the indicated concentrations of nonlipoprotein cholesterol dissolved in ethanol (10 mg cholesterol/ml ethanol). After 24 h, cells were washed, incubated for 1 h at 37°C with medium containing 5 μg ^{125}I-HDL$_3$ protein/ml, and HDL binding was determined as described in Methods. The mean protein content per dish for fibroblasts and smooth muscle cells was 121 and 38 μg/dish, respectively, and was unaffected by the medium cholesterol concentration. These results are representative of eight similar experiments using four different strains of fibroblasts and two strains of smooth muscle cells. Incubation with medium containing ethanol alone (up to 1%) had no effect on subsequent binding of ^{125}I-HDL$_3$ to cells (data not shown).

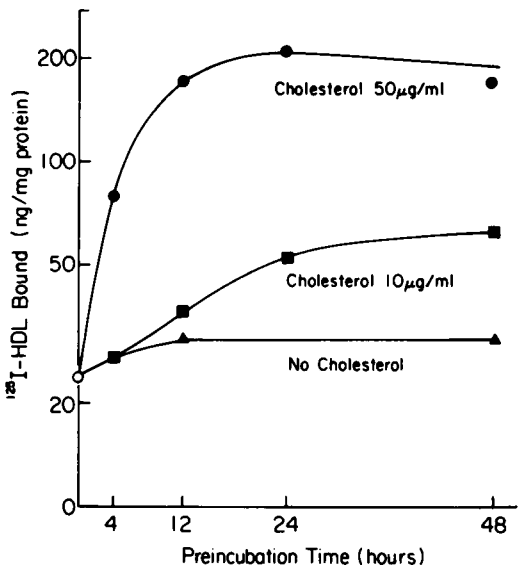

FIGURE 2 Time course of the effect of nonlipoprotein cholesterol on ^{125}I-HDL$_3$ binding to fibroblasts. Fibroblasts were incubated for the indicated periods of time with serum-free medium containing albumin plus zero (▲), 10 μg/ml (■), or 50 μg/ml (●) cholesterol. Binding of ^{125}I-HDL$_3$ was then measured as described for Fig. 1.

Exposure of fibroblasts to cholesterol resulted in rapid stimulation of ^{125}I-HDL$_3$ binding, which was sustained for at least 48 h (Fig. 2). This stimulation of HDL$_3$ binding appeared to involve synthesis of protein. When cycloheximide was added to the medium to inhibit protein synthesis, cholesterol-mediated enhancement of HDL$_3$ binding to both fibroblasts and arterial smooth muscle cells was arrested after the first few hours of exposure to cholesterol (Fig. 3).

The increase in HDL$_3$ binding to fibroblasts induced by preincubation with cholesterol was readily reversed when cells were incubated with cholesterol-free medium (Fig. 4). Previous studies showed that HDL$_3$ promoted cholesterol efflux from fibroblasts, while HDL$_2$ tended to promote net delivery of cholesterol into cells (1, 4, 10, 18). When HDL$_3$ was added to the cholesterol-free medium ^{125}I-HDL$_3$ binding was reduced at a faster rate (Fig. 4). In contrast, reduction of ^{125}I-HDL$_3$ binding was slowed in the presence of HDL$_2$. Thus, reversal of HDL binding activity was accelerated in the presence of specific lipoprotein particles that act as acceptors for cellular cholesterol.

To determine if intracellular delivery of lipoprotein sterol could also affect HDL binding, fibroblasts were preincubated with variable concentrations of LDL after first being exposed to LDS to induce LDL receptor activity. To test for intracellular delivery of cholesterol, two well-characterized parameters of cell

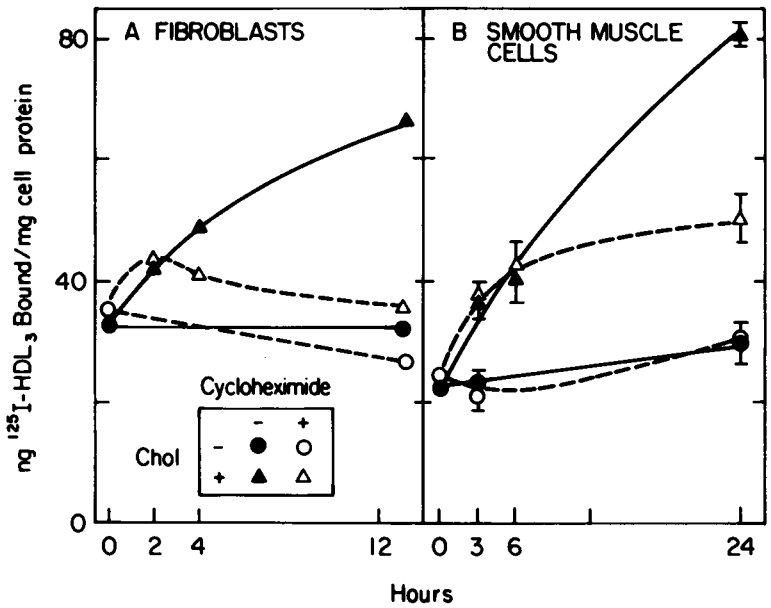

FIGURE 3 The effects of cycloheximide on enhancement by nonlipoprotein cholesterol of ^{125}I-HDL$_3$ binding to fibroblasts (*A*) and arterial smooth muscle cells (*B*). Cells were incubated for the times indicated with serum-free, albumin medium plus no additions (●), 0.5 mM cycloheximide (○), 50 μg/ml cholesterol (▲), or cycloheximide plus cholesterol (△). Binding of ^{125}I-HDL$_3$ was then measured as described in Fig. 1. For dishes receiving cycloheximide-containing medium (○, △), an aliquot of 50 mM cycloheximide was added directly to the growth medium (0.5 mM final concentration) 2 h before the change to the binding medium. Values for panel *A* represent the mean of duplicate dishes while values for panel *B* represent the mean±SEM of four dishes.

cholesterol metabolism, cholesterol synthesis, and cholesterol esterification, were measured simultaneously with ^{125}I-HDL$_3$ binding. Increasing the medium LDL concentration enhanced both ^{125}I-HDL$_3$ binding and incorporation of [^{14}C]oleate into cholesteryl esters, while the rate of cholesterol synthesis from [^{14}C]oleate was decreased (Fig. 5). The effects of LDL preincubation on HDL binding and cholesterol esterification became maximum at an LDL concentration reported to cause saturation of LDL-receptor binding (19, 20). A direct comparison of HDL binding and cholesterol esterification revealed a linear relationship (Fig. 5, *inset*), suggesting that the activities of both processes were mutually regulated in response to increased intracellular delivery of LDL sterol.

Direct evidence that HDL binding is regulated in response to changes in the intracellular cholesterol content was provided by studies showing that the ability of cells to bind HDL was proportional to the amount of unesterified cholesterol mass in the cell. When fibroblasts were preincubated with medium containing different concentrations of either nonlipoprotein cholesterol or LDL, HDL binding to cells increased in parallel to the cell unesterified cholesterol content (Fig. 6). The relationship between HDL binding and cellular unesterified cholesterol was linear, despite the use of two different methods to transport cholesterol into cells. In contrast, mass measurements did not reveal any correlation between HDL binding and the cell esterified cholesterol content (data not shown). This was apparently related to the observation that cells accumulated more esterified cholesterol in the presence of LDL (10–20% of total sterol) than in the presence of nonlipoprotein cholesterol (<5% of total sterol), regardless of the final unesterified cholesterol content. It is possible that a substantial proportion of the cellular cholesterol ester in the LDL-treated cells represented vesicular and lysosomal pools of unhydrolyzed LDL sterol, since cells were preincubated with LDS in order to induce LDL receptors and promote rapid endocytosis of LDL particles.

Additional evidence that HDL$_3$ binding to fibroblasts was regulated in response to changes in the cell cholesterol content was provided by studies comparing HDL binding activity with the activities of other biochemical processes known to be involved in modulation

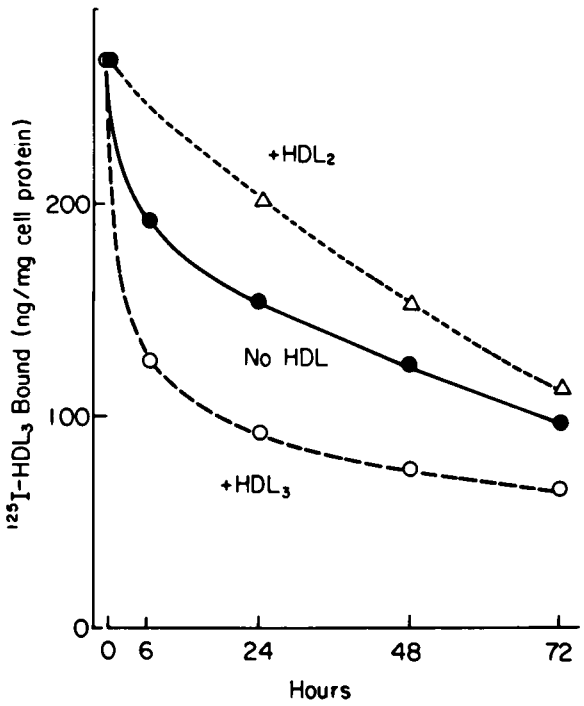

FIGURE 4 Reversal of ^{125}I-HDL$_3$ binding to fibroblasts previously exposed to cholesterol-rich medium. Fibroblasts were incubated with serum-free, albumin medium containing 50 μg cholesterol/ml. After 48 h, cells were washed five times with PBS containing 2.0 mg/ml albumin and then incubated with serum-free, albumin medium alone (●) or albumin medium plus 50 μg protein/ml HDL$_3$ (○) or HDL$_2$ (△). The HDL$_2$ had been treated to remove apo B, E as described in Methods. After the times indicated, ^{125}I-HDL$_3$ binding was measured as described for Fig. 1. For zero hour values, the albumin medium was added to dishes and removed immediately.

of cell cholesterol homeostasis. To induce changes in the activities of these biochemical processes, fibroblasts were incubated under conditions designed to vary the rate of flux of cholesterol into or out of the cell. To promote removal of cholesterol from cells, HDL$_3$ was added to the medium. As a lipoprotein control that has little effect on net cholesterol transport, HDL$_2$ was added to the medium. To promote different rates of cholesterol delivery into cells, fibroblasts were incubated with medium containing different concentrations of either nonlipoprotein cholesterol or LDL. After 48 h of incubation under these conditions, cells were assayed for ^{125}I-HDL$_3$- and ^{125}I-LDL-binding activities and rates of cholesterol synthesis and esterification. Results demonstrated that HDL$_3$ binding was inversely correlated with ^{125}I-LDL binding (Fig. 7 *A*) and with the rate of cholesterol synthesis (Fig. 7 *B*) but positively correlated with the rate of cholesterol esterification (Fig. 7 *C*). With the addition of HDL$_3$ to the medium, LDL receptor activity and the rate of cholesterol synthesis were both increased, while cholesterol esterification and HDL binding were both slightly decreased. In contrast, addition of HDL$_2$ to the medium had little effect on any of the processes measured. Addition of the different concentrations of either nonlipoprotein cholesterol or LDL led to varying degrees of enhancement of both HDL binding and cholesterol ester formation and to suppression of both LDL binding and the rate of cholesterol synthesis.

The relationship between HDL$_3$ binding and both LDL binding and the rate of cholesterol synthesis was

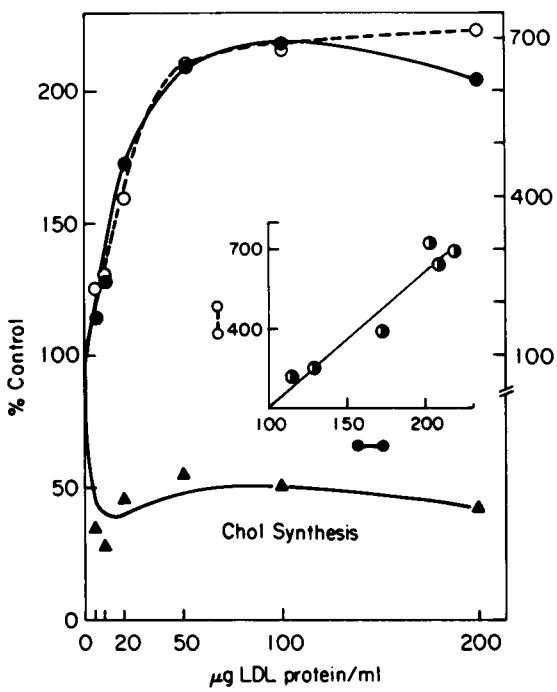

FIGURE 5 Effects of preincubation with LDL on ^{125}I-HDL$_3$ binding, cholesterol synthesis, and cholesterol esterification by fibroblasts. Fibroblasts were preincubated with medium containing 10% LDS to induce the LDL receptor. After 24 h, fresh medium was added. After another 24 h, cells were incubated with serum-free, albumin medium containing the indicated concentration of LDL protein. After an additional 24 h, half of the dishes were assayed for ^{125}I-HDL$_3$ binding (●) as described in Fig. 1 while the other half received medium containing [^{14}C]oleic acid, and incorporation of radioactivity into unesterified (▲) and esterified (○) cholesterol was measured as described in Methods. The *inset* shows the relationship between ^{125}I-HDL$_3$ binding and cholesteryl [^{14}C]oleate formation. The control (100%) values per milligram cell protein for HDL binding, cholesterol esterification, and cholesterol synthesis were 47.2 ng HDL$_3$, 791 pmol oleate, and 81 pmol oleate, respectively.

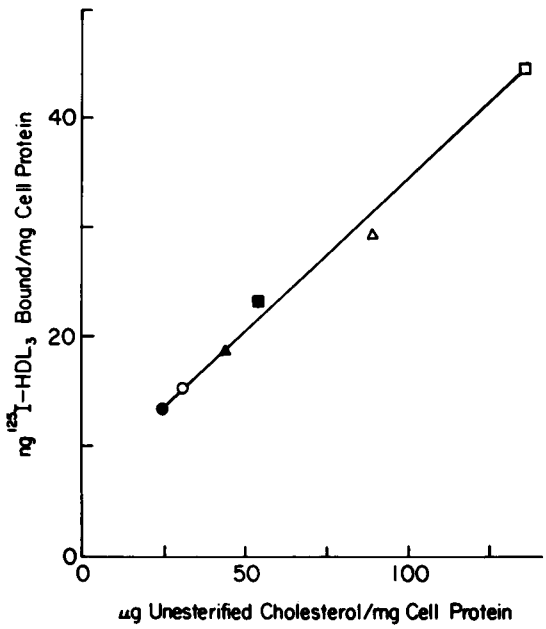

FIGURE 6 Comparison of ^{125}I-HDL$_3$ binding to fibroblasts with the cell cholesterol content after preincubation with medium containing nonlipoprotein cholesterol or LDL. Before assays for HDL binding and cell sterol content, fibroblasts were incubated in parallel according to two different protocols. For protocol A, cells were incubated for 48 h with serum-free medium containing 0.2% albumin plus either zero (○), 20 (△), or 50 (□) μg cholesterol dissolved in ethanol. For protocol B, cells were preincubated for 48 h with medium containing LDS as described for Fig. 5 followed by an additional 24-h incubation with serum-free medium containing 0.2% albumin plus either zero (●), 10 μg (▲), or 50 μg (■) LDL protein/ml. For measurement of cell sterol content, half of the dishes were chilled on ice, washed four times with PBS-albumin medium and twice with PBS, and cell sterols were extracted as described in Methods. For measurement of HDL binding, the other set of dishes was washed, incubated at 37°C with ^{125}I-HDL$_3$, and assayed for cell-associated ^{125}I-HDL$_3$ as described for Fig. 1. Each data point represents six incubations: three each for HDL binding and for cellular cholesterol values. The standard error of the mean for each value was <10% of the mean.

hyperbolic; that is, the largest increases in HDL$_3$ binding occurred at cholesterol concentrations that almost totally suppressed both of the other processes (Fig. 7 *A* and *B*). On the other hand, the relationship between HDL$_3$ binding and cholesterol esterification was linear (Fig. 7 *C*), providing additional evidence that these two processes are mutually regulated.

As reported previously (10), most of the HDL associated with cells after short-term incubations at 37°C is apparently bound to cell-surface sites rather than internalized. To ascertain that exposure of cells to cholesterol enhances binding of HDL to cell-surface sites, the ^{125}I-HDL$_3$-binding assay was performed at 0°C to prevent surface-bound HDL from being internalized by the cells. The absolute amount of ^{125}I-HDL$_3$ bound after 0°C incubation was markedly less than that bound after 37°C incubation (Table I). The reason for this large temperature effect is unclear; however, a similar effect on LDL binding to fibroblasts has been reported (20). Despite this large absolute difference in binding, addition of cholesterol to the preincubation medium caused increases in HDL$_3$ binding of similar magnitudes at both temperatures (Table I). Therefore, it is likely that the cholesterol-induced increase in HDL binding observed after 37°C incubations was related to an increase in binding to sites on the cell surface.

Saturation curves for ^{125}I-HDL$_3$ binding to the cell surface of fibroblasts revealed that preincubation with cholesterol led to an increase in the high-affinity, saturable component of HDL binding (Fig. 8). Scatchard plots (21) of these saturation curves demonstrated that this increased HDL binding was largely due to an increase in the apparent maximum capacity of the high-affinity component (Fig. 8, *inset*), indicative of an increase in the number of binding sites. In the up-regulated state, the apparent K_d for HDL binding at 0°C was 2 μg protein/ml.

To assess specificity of the induced HDL binding sites, the ability of unlabeled lipoprotein subfractions to compete for ^{125}I-HDL$_3$ binding was tested. At up to 20-fold protein excess, LDL had only a slight ability to compete for ^{125}I-HDL$_3$ binding (Fig. 9). In contrast, HDL$_3$ and HDL$_2$ blocked binding of ^{125}I-HDL$_3$ by more than two-thirds. Thus, the binding sites induced by cholesterol appeared to be specific for HDL but did not distinguish between the major HDL subfractions.

At similar medium concentrations, HDL is inter-

TABLE I
Comparison of the Effects of Preincubation with Nonlipoprotein Cholesterol on Binding of ^{125}I-HDL$_3$ at 37° and 0°C

	^{125}I-HDL$_3$ bound		
	Control	+Cholesterol	Percent control
	ng/mg cell protein		
37°C	75.0	286	381
0°C	12.4	61.4	495

Fibroblasts were preincubated for 24 h with medium containing no cholesterol (control) or 100 μg/ml cholesterol added in ethanol (10 μl/ml). Binding of ^{125}I-HDL$_3$ (5 μg protein/ml) was then measured at either 0° or 37°C as described in Methods.

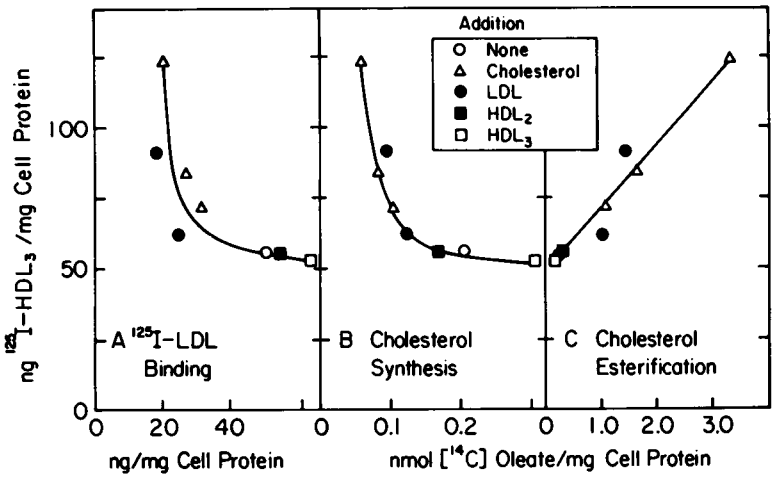

FIGURE 7 A comparison of ^{125}I-HDL$_3$ binding to fibroblasts with other biochemical parameters of cell cholesterol metabolism in response to preincubation with nonlipoprotein cholesterol and different lipoprotein subfractions. Fibroblasts were incubated with serum-free medium containing 0.2% albumin plus either no additions (○), three concentrations (10, 20, 50 μg/ml) of nonlipoprotein cholesterol (△), two concentrations (20 and 200 μg protein/ml) of LDL (●), 50 μg protein/ml HDL$_2$ (■), or 50 μg protein/ml HDL$_3$ (□). After 48 h, ^{125}I-HDL$_3$ binding was measured as described in Fig. 1, cholesterol synthesis (panel *B*), and cholesterol esterification (panel *C*) were measured as described in Fig. 5, and ^{125}I-LDL binding (panel *A*) was measured as described in Methods.

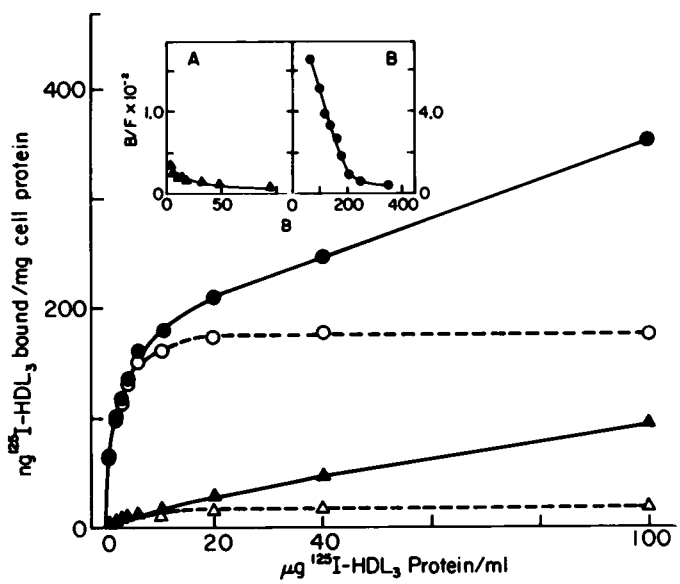

FIGURE 8 Effect of nonlipoprotein cholesterol on saturation curves for ^{125}I-HDL$_3$ binding to fibroblasts at 0°C. Fibroblasts were incubated with serum-free, albumin medium containing no cholesterol (▲, △) or 50 μg cholesterol/ml (●, ○). After 24 h, cells were washed, chilled to 0°C, incubated with the indicated concentration of ^{125}I-HDL$_3$, and assayed for HDL binding as described in Methods. Values for "high-affinity" binding (△, ○) were calculated by subtraction of "low affinity" binding at each HDL concentration as estimated from the slope of the linear component of total binding. The *inset* shows Scatchard plots of total binding where B represents nanograms HDL bound per milligram cell protein, and F represents the concentration of HDL in the medium in nanograms per milliliter.

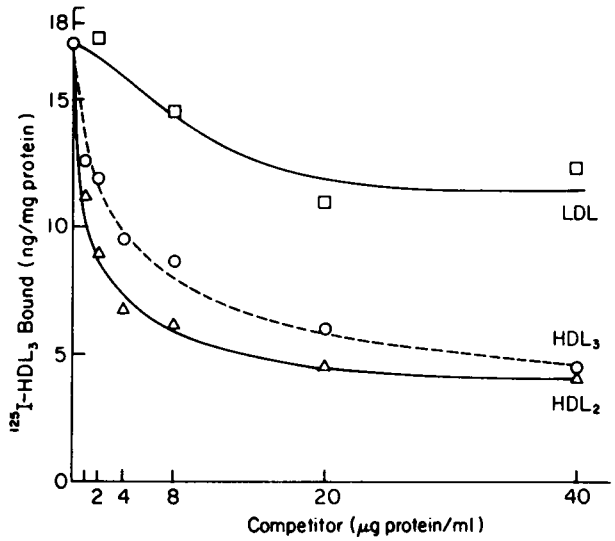

FIGURE 9 Competition for ^{125}I-HDL$_3$ cell binding at 0°C by HDL$_3$, HDL$_2$, and LDL after preincubation of fibroblasts with nonlipoprotein cholesterol. Fibroblasts were preincubated with medium containing 50 μg cholesterol/ml. After 24 h, cells were washed, chilled to 0°C, and incubated with medium containing 2 μg ^{125}I-HDL$_3$/ml plus the indicated concentration of unlabeled LDL (□), HDL$_3$ (○), or HDL$_2$ (△). Binding of ^{125}I-HDL$_3$ was measured as described in Methods.

nalized and degraded by cultured extrahepatic cells at a much slower rate than is LDL (22–24). As recently reported (10), most of the HDL bound to fibroblasts at 4°C is subsequently released intact into the medium upon warming the cells to 37°C. Similar results were seen when binding was up-regulated by preincubation with cholesterol (Fig. 10). When the ^{125}I-HDL$_3$-binding assay was performed at 4°C, most of the cell-associated ^{125}I was released when cells were treated with trypsin (Fig. 10 *A* and *B*). When medium containing unlabeled HDL$_3$ was added and the dishes were warmed to 37°C, >80% of the trypsin-releasable ^{125}I was displaced from the cells within 1 h. In contrast, when cells were maintained at 4°C, ^{125}I-HDL$_3$ was displaced from the cells at a much slower rate, with 50–60% of the initial bound HDL still associated with cells after 4 h. Most of the displaced ^{125}I was recovered in the medium as TCA-insoluble derivatives (Fig. 10 *C*), suggesting that release of intact HDL particles from the cell surface accounted for the displacement of prebound HDL. Virtually none of the ^{125}I-HDL$_3$ was internalized (Fig. 10 *B*) or degraded (Fig. 10 *D*) by the cells. Apparently, cell-surface binding of HDL is reversible, and the rate of displacement of prebound HDL is temperature dependent.

To directly test the hypothesis that HDL binding to cells functions to facilitate removal of cholesterol from the cell, both ^{125}I-HDL$_3$ binding and [^{3}H]cholesterol efflux were measured simultaneously. Fibroblasts were preincubated with 50 μg/ml [^{3}H]cholesterol to up-regulate HDL binding and to radiolabel cellular cholesterol and then incubated for 4 h with medium containing increasing concentrations of either unlabeled HDL$_3$ or ^{125}I-HDL$_3$ (Fig. 11). Results demonstrated that the saturation curves for both [^{3}H]cholesterol efflux from cells and ^{125}I-HDL$_3$ binding had similar profiles, with both processes revealing a high-affinity component that saturated at an HDL$_3$ concentration of 20 μg HDL$_3$ protein/ml (Fig. 11 *A*). Measurements of cholesterol mass in the cell produced

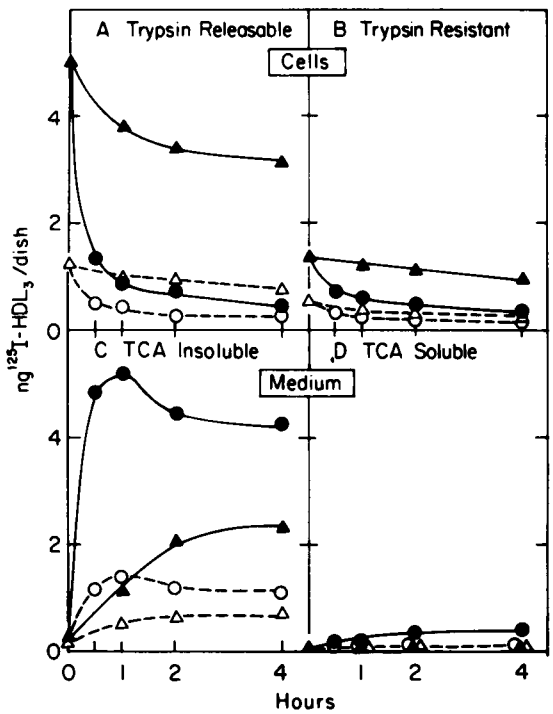

FIGURE 10 Fate of ^{125}I-HDL$_3$ bound to fibroblasts at 4°C after subsequent incubations at 4° or 37°C. Fibroblasts were preincubated with albumin medium containing no cholesterol (○, △) or 50 μg cholesterol/ml (●, ▲). After 48 h, cells were washed, chilled to 0°–4°C, and incubated with 10 μg/ml ^{125}I-HDL$_3$. After 2 h, cells were washed seven times by the standard procedure described in Methods, and cold serum-free, albumin medium containing 10 mM Hepes (pH 7.4) and 10 μg/ml unlabeled HDL$_3$ was added to the dishes. One set of dishes was then warmed to 37°C (○, ●) while the other set was maintained at 4°C (△, ▲). After the times indicated, the medium was collected and treated with TCA. TCA-insoluble (panel *C*) and TCA-soluble (panel *D*) radioactivity was measured. The monolayers were then washed twice with PBS and then trypsinized, and trypsin-releasable (panel *A*) and trypsin-resistant (panel *B*) radioactivity was measured as described in Methods. For zero time value, cold chase medium was added and immediately removed.

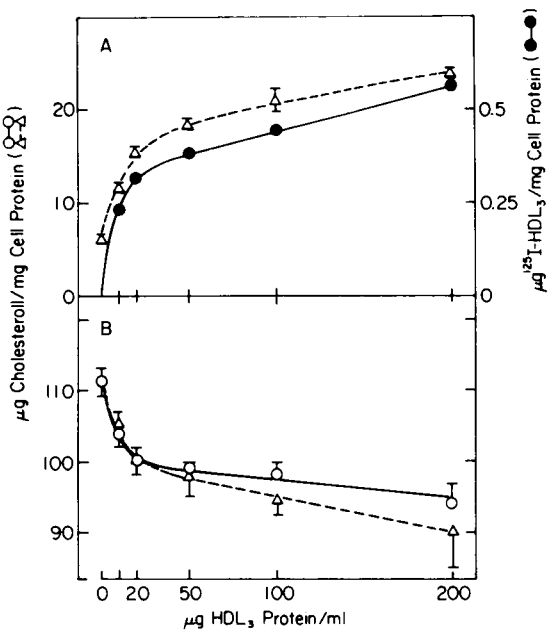

FIGURE 11 Comparison of ^{125}I-HDL$_3$ binding to fibroblasts with promotion of cholesterol efflux from cells by HDL$_3$. Fibroblasts were preincubated with serum-free, albumin medium containing 50 μg [^{3}H]cholesterol/ml (0.5 μCi/ml). After 48 h, cells were washed five times as described for Fig. 4 and incubated with serum-free, albumin medium containing the indicated concentrations of either unlabeled HDL$_3$ (○, △) or ^{125}I-HDL$_3$ (●). After 4 h, the dishes were chilled to 0°C and the medium from dishes containing unlabeled HDL$_3$ was collected for [^{3}H]cholesterol determinations (panel *A*, △). All dishes were then washed, and cells were assayed for ^{125}I-HDL$_3$ binding (panel *A*, ●), [^{3}H]cholesterol content (panel *B*, △), or unesterified cholesterol mass (panel *B*, ○) as described in Methods. The [^{3}H]cholesterol content of the cells and medium was converted to mass equivalents using the mean value for the specific activity of unesterified cholesterol in cells incubated in the absence of HDL$_3$. Each value represents the mean of duplicate and quadruplicate (±SEM) incubations for HDL binding and cholesterol content, respectively. The cell esterified cholesterol content was <2% of the total sterol content (data not shown).

results nearly identical to those obtained from measurements of cellular [^{3}H]cholesterol (Fig. 11 *B*), indicating that HDL$_3$ promoted net transport of cholesterol from cells rather than exchange of unlabeled lipoprotein cholesterol for radiolabeled cellular cholesterol. These results demonstrate a close association between binding of HDL$_3$ to its high-affinity cell-surface sites and its effects on cholesterol transport from the cell.

DISCUSSION

Recent results from our laboratory (10) demonstrated that cultured human skin fibroblasts and human arterial smooth muscle cells possess cell-surface high-affinity binding sites specific for HDL. Results from the current study indicate that HDL binding to these sites is regulated in response to cholesterol loading of cells. Binding of ^{125}I-HDL$_3$ to both fibroblasts and smooth muscle cells was enhanced by preincubation with medium containing nonlipoprotein cholesterol. This enhancement was dose dependent and was sustained for at least 48 h in the presence of cholesterol. When the cholesterol-enriched medium was replaced with a cholesterol-free medium, HDL binding to fibroblasts returned to basal levels, indicating that the effects of cholesterol were reversible. The rate of reversal of HDL binding activity was enhanced when an acceptor for cellular cholesterol, such as HDL$_3$ (1, 18), was added to the medium. Thus, the HDL binding activity of cells appears to change in parallel with changes in the availability of exogenous cholesterol.

Tauber et al. (25) demonstrated that treatment of bovine endothelial cells with 25-hydroxycholesterol resulted in enhanced binding of HDL to cells. Our studies also showed that 25-hydroxycholesterol was a potent activator of HDL binding to both fibroblasts and arterial smooth muscle cells (data not shown). However, at concentrations required to produce consistent effects (>20 μg/ml), 25-hydroxycholesterol was markedly cytotoxic to both cell types. Nonlipoprotein cholesterol, however, had no apparent cytotoxic effects at concentrations up to 100 μg/ml. Since gas-liquid chromatography failed to reveal detectable amounts of 25-hydroxycholesterol or other oxygenated sterols in the cholesterol preparations used in the present study, it is likely that the observed stimulatory effects of cholesterol were due to cholesterol itself rather than oxygenated sterol contaminants.

Preincubation of fibroblasts with medium containing lipoprotein cholesterol in the form of LDL also resulted in increased binding of HDL$_3$ to fibroblasts. Evidence suggests that enhancement of HDL$_3$ binding by treatment of cells with both nonlipoprotein and LDL cholesterol occurred by the same mechanism related to delivery of cholesterol into the cells. First, HDL$_3$ binding was directly proportional to the unesterified cholesterol content of the cell when fibroblasts were exposed to medium containing different concentrations of either nonlipoprotein or LDL cholesterol. Second, after cells were incubated with media of different lipoprotein and nonlipoprotein cholesterol composition, HDL binding activity was inversely correlated with both LDL receptor activity and the rate of cholesterol synthesis but positively correlated with the rate of cholesterol ester formation. Thus, HDL binding activity was regulated in concert with the activities of other biochemical processes known to be affected by changes in cell cholesterol content (19, 26). Compar-

ison of HDL binding activity to the rate of cholesterol ester formation revealed a positive linear relationship, suggesting that the regulatory mechanisms for these two processes are closely coupled.

When compared to preincubation with nonlipoprotein cholesterol, preincubation with LDL had only a limited ability to enhance HDL_3 binding to fibroblasts. The maximum effects of LDL treatment corresponded to the effects observed in the presence of 10–20 μg/ml nonlipoprotein cholesterol. Presumably this reflects the fact that intracellular delivery of LDL cholesterol is a receptor-mediated, saturable process that undergoes down-regulation when cells accumulate cholesterol (19). This conclusion was supported by results showing that the effects of LDL on both HDL binding and cholesterol esterification became maximum at an LDL concentration of ~50 μg protein/ml, similar to that reported to cause saturation of LDL receptor binding (19, 20). In contrast, the effects of nonlipoprotein cholesterol on HDL binding and cholesterol esterification did not completely saturate at concentrations up to 100 μg/ml (data not shown), probably because cholesterol in this form enters cells by diffusion.

Several different lines of evidence suggest that exposure of cells to cholesterol-rich medium leads to induction of cell-surface binding sites specific for HDL. First, the stimulatory effect of cholesterol treatment on HDL binding to fibroblasts and smooth muscle cells was largely prevented by cycloheximide, implying that synthesis of new protein was required for the enhanced binding. Second, preincubation with cholesterol stimulated HDL binding to fibroblasts even when the binding assay was performed at 0°C to prevent internalization of surface-bound particles, indicating that cholesterol specifically enhanced binding to HDL to sites on the cell surface. Third, kinetic analysis of the saturation curves of ^{125}I-HDL_3 binding to fibroblasts at 0°C revealed that exposure of cells to cholesterol caused a severalfold increase in the apparent maximum capacity of the high-affinity binding sites, indicative of an increase in the number of sites. Scatchard (21) analysis of up-regulated high-affinity binding yielded linear plots with an apparent K_d of ~2 μg HDL protein/ml. Results from competitive binding studies showed that binding sites induced by preincubation with cholesterol were relatively specific for HDL; that is, both unlabeled HDL_3 and HDL_2 were effective competitors for ^{125}I-HDL_3 binding to fibroblasts while LDL was a much weaker competitor. Studies designed to identify more specifically the HDL binding ligand are currently being conducted in our laboratory.

Results from this study provide further support for the hypothesis that promotion of cholesterol efflux from cells is facilitated by binding of HDL to its high-affinity, cell-surface-binding sites (10, 18). When fibroblasts were pretreated with [^{3}H]cholesterol both to enhance HDL binding and to radiolabel cellular cholesterol, promotion of [^{3}H]cholesterol efflux from cells from HDL_3 during subsequent short-term incubations had a high-affinity, saturable component, which was nearly identical in profile to that observed for high-affinity HDL binding. This high-affinity efflux component was associated with a decrease in cholesterol mass in the cell, indicating that it represented net transport of cholesterol from cells. These results are in agreement with previous results from our laboratory (10, 18) showing that the effects of HDL_3 on parameters of cell cholesterol metabolism were saturable, becoming maximum at an HDL concentration of ~20 μg/ml. Daniels et al. (3) reported similar saturability in the capacity of sterol-depleted HDL to promote cholesterol efflux from fibroblasts.

Additional support for the hypothesis that the induced HDL binding sites on fibroblasts function to facilitate cholesterol transport from cells was provided by results showing that most of the ^{125}I-HDL_3 bound to these sites at 4°C was rapidly released into the medium upon subsequent warming of the cells to 37°C. The released radioactivity appeared to represent HDL particles that were not substantially degraded by cells, since virtually all of it was precipitable by TCA treatment of the medium. Only a negligible amount of the prebound ^{125}I-HDL_3 was internalized or completely degraded by cells. Rapid dissociation of HDL from its binding sites is consistent with the proposal that the role of HDL is to transport cholesterol from extrahepatic cells rather than deliver cholesterol into these cells by an endocytotic process.

Results from this study provide strong evidence that the HDL binding sites on human fibroblasts and human arterial smooth muscle cells represent true receptors, which may play an important role in modulation of cell cholesterol homeostasis. In addition, through the course of the present study, evidence emerged that allowed for further characterization of the functional relationship between the different biochemical processes involved in cell cholesterol metabolism. The largest degree of regulation of LDL binding and cholesterol synthesis occurred over a range of apparent cell cholesterol content below which both HDL binding and cholesterol esterification were nearly completely suppressed (Fig. 7). Conversely, the largest degree of regulation of HDL binding and cholesterol esterification occurred over an apparent range of cell cholesterol content above which LDL binding and cholesterol synthesis was suppressed. These results suggest that regulation of LDL receptor activity (exogenous

cholesterol supply) and cholesterol synthesis (endogenous supply) functions to protect cells from overdepletion of cholesterol, while regulation of HDL receptor activity (exogenous removal) and cholesterol esterification (endogenous removal) functions to protect cells from overaccumulation of unesterified cholesterol. This apparent protective function of the HDL receptor may be related to the inverse correlation between serum HDL cholesterol levels and the incidence of atherosclerosis (27), a disease characterized by excessive accumulation of cholesterol in cells of the arterial wall.

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