High density lipoprotein (HDL) binding to human fibroblasts and arterial smooth muscle cells is up-regulated when sterol is delivered to cells in the form of nonlipoprotein cholesterol or low density lipoprotein (LDL). Results from the present study show that the HDL binding activity of aortic endothelial cells is up-regulated when cholesterol in the form of acetylated LDL (AcLDL) is delivered to cells via the "scavenger" lipoprotein receptor pathway. AcLDL treatment led to a dose-dependent, but saturable, increase in HDL binding to cultured bovine aortic endothelial cells that was reversed when cells were treated with lipoprotein-deficient serum. The AcLDL-mediated enhancement in HDL binding activity was inhibited by cycloheximide, suggesting the involvement of protein synthesis. This enhancement was associated with an increased cell cholesterol content, a suppressed rate of cholesterol synthesis, and an increased rate of cholesterol ester formation. Kinetic analysis of HDL binding showed that AcLDL treatment caused an increase in the apparent number of high-affinity binding sites (Kd ~ 3 μg/ml HDL protein). Competition and direct binding studies revealed that the inducible binding sites exhibited relative specificity for HDL over LDL and AcLDL. Thus, aortic endothelial cells appear to possess specific receptors for HDL that may function to facilitate HDL-mediated removal from cells of excess cholesterol internalized by the scavenger receptor pathway.
Cells

Bovine aortic endothelial cells were obtained and cultivated as described by Schwartz,15 and human skin fibroblasts were grown as previously described10 using 10% fetal bovine serum in either Waymouth's or modified Dulbecco-Vogt medium. Cells from between the fourth and twelfth passage were seeded in 35 mm dishes at a density of approximately 150,000 (endothelial cells) or 50,000 (fibroblasts) cells per dish. After 7 to 15 days, when the cells were quiescent, the cells were washed twice with either phosphate-buffered saline (PBS) or Dulbecco-Vogt medium (containing 2 mg/ml of bovine serum albumin) and then were incubated with either Waymouth's or Dulbecco-Vogt medium containing 2 mg/ml albumin (medium A) plus the additions indicated in the figure legends.

Lipoproteins and Lipoprotein-Deficient Serum

Lipoproteins were isolated from human serum by sequential ultracentrifugation16 at the following densities: LDL, d = 1.019–1.063; HDL, d = 1.125–1.21. LDL and HDL were iodinated with 125I by the method of McFarlane as modified by Bilheimer et al.17 with previously described results.10 LDL was acetylated by the method of Goldstein et al.13 Effective acetylation was confirmed by agarose electrophoresis of the modified and starting LDL preparations. Lipoproteins were quantified according to their protein content as determined by the method of Lowry et al.18 Lipoprotein-deficient serum (LDS) consisted of the d > 1.25 serum fraction.10

Methods

Binding of 125I-HDL and 125I-LDL

Binding of 125I-HDL to cells was determined as previously described.4 Briefly, cells were washed rapidly three times with PBS containing 2 mg/ml albumin (wash buffer) and then incubated with binding medium at either 37° C or 4° C. For the 37° assay, 125I-HDL was added to medium A at a concentration of 5 µg protein/ml. For the 4° C assay, the medium was buffered with 20 mM HEPES instead of sodium bicarbonate (medium B). After either 1 hour at 37° C or 2 hours at 4° C, the binding medium was removed and the cells were washed seven times at 4° C with wash buffer (the first five with albumin and the last two without albumin). Preliminary experiments showed these binding incubation times to be beyond the initial rapid rise in HDL binding. The cells were then digested in 0.1 N NaOH and aliquots were taken for assays of 125I and protein.18 Binding of 125I LDL was determined using the dextran-sulfate method previously described.10 Over 50% of the total cell-associated 125I-LDL was releasable by dextran sulfate. In studies of the fate of prebound 125I-HDL, the chase medium was treated with trichloroacetic acid (10% final concentration) to assess the release of degradation products from the cells.4

Cholesterol Synthesis, Esterification, and Mass

The rate of esterification of cholesterol was estimated by assessing the rate of incorporation of 14C-oleate into cholesteryl ester. The rate of synthesis of cholesterol was determined in the same cells by measuring the rate of incorporation of 14C-oleate (via

Figure 1. Effects of AcLDL on HDL binding activity of bovine endothelial cells (EC) and human fibroblasts (FB). Cultured cells were incubated with serum-free culture medium containing 2 mg/ml albumin (medium A, see Methods) and the indicated concentration of AcLDL. After 48 hours HDL binding activity was determined by the 37° C pulse-incubation assay described in Methods. The data in the inset are from a separate experiment. The 100% values for the inset were 16.9 ng (EC) and 26.0 ng (FB) 125I-HDL bound per milligram of cell protein.

Figure 2. A comparison of the effects of AcLDL on HDL binding activity, rate of cholesterol esterification, and rate of cholesterol synthesis in endothelial cells. Cells were incubated with the indicated concentration of AcLDL as described for Figure 1. After 48 hours, HDL binding activity and rates of cholesterol esterification and synthesis were determined by the 37° C pulse-incubation assay described in Methods.
14C-acetyl units) into unesterified cholesterol as validated previously.10 Briefly, after the indicated treatment, cells were washed twice with phosphate-buffered saline at room temperature and incubated with medium A containing 14C-oleic acid (20 μM to 30 μM, 2 μCi/ml) bound to albumin (0.05% to 0.10%). After 1 hour at 37 °C, cells were chilled on ice and washed twice with ice-cold wash medium. Sterols were extracted in hexane-isopropanol, isolated by thin-layer chromatography, and quantified by scintillation counting as described previously.10 Cellular esterified and unesterified cholesterol mass was measured by the method of Heider and Boyett19 after extraction and thin-layer chromatography of the lipids.

**Results**

Treatment of bovine aortic endothelial cells with acetylated LDL (AcLDL) led to a dose-dependent increase in the binding of HDL3 to the cells. This increase saturated at an AcLDL concentration of approximately 20 μg protein/ml (Figure 1). This would be the expected saturation profile if the effect of AcLDL on HDL binding activity were mediated by the binding of AcLDL to the scavenger receptor.13 Support for this conclusion was provided by studies showing that AcLDL has little effect on the binding of HDL to human fibroblasts, a cell type that lacks scavenger receptors (Figure 1 inset). The inability of AcLDL to stimulate HDL binding to fibroblasts was observed even when treatment of cells with 50 μg/ml of nonlipoprotein cholesterol caused a sixfold increase in HDL binding (data not shown). Concurrent with the increase in HDL binding activity, treatment of endothelial cells with AcLDL led to a suppression of the rate of cholesterol synthesis and an increase in the rate of cholesteryl ester formation (Figure 2).

Typically, incubation of endothelial cells with medium containing AcLDL was associated with a gradual increase in HDL binding activity for up to 48 hours, although the activation time course varied in different experiments (see Figures 3 and 4). This enhancement of HDL binding activity accompanied an increase in the cellular content of both esterified and unesterified cholesterol mass (Figure 5). The time-dependent stimulation of HDL binding activity by AcLDL was arrested when cycloheximide was added to the medium, suggesting that protein synthesis was involved (Figure 3).

The AcLDL-mediated increase in HDL binding activity was readily reversed when cells were exposed to sterol-deficient medium (Figure 4). However, this reversibility required the presence of serum proteins.
Figure 5. The effects of time of incubation with AcLDL on the unesterified and esterified cholesterol content of endothelial cells. Cells were incubated with medium A containing zero (○,△) or 25 μg/ml (●,▲) AcLDL. At the times indicated, cells were chilled on ice and thoroughly washed, and sterols were extracted and quantified as described in Methods.

Figure 6. The fate of HDL bound to endothelial cells after subsequent incubation at 4°C or 37°C. Cells were incubated with medium A plus 25 μg/ml AcLDL. After 48 hours, cells were washed, chilled to 4°C, and incubated with medium B (see Methods) plus 5 μg/ml 125I-HDL3. After 2 hours, cells were washed seven times as described in Methods for the binding assay procedure; cold medium B containing 5 μg/ml unlabeled HDL3 was added to the dishes. One set of dishes was then warmed to 37°C (closed symbols), while another set was maintained at 4°C (open symbols). After the times indicated, the medium was collected and treated with trichloroacetic acid (TCA), and TCA-insoluble and TCA-soluble radioactivity was measured (B). The monolayers were then washed twice with phosphate-buffered saline and dissolved in 0.1 N NaOH for determination of cell-associated radioactivity (A).

Figure 7. The effects of AcLDL treatment on saturation curves of 125I-HDL3 and 125I-LDL binding to endothelial cells. Cells were incubated with medium A containing either zero (○,△) or 50 μg/ml (●,▲) AcLDL. After 72 hours, cells were washed and incubated at 37°C with medium A containing the indicated concentrations of either 125I-HDL3 or 125I-LDL. After 1 hour, dishes were chilled to 4°C and washed seven times as described in Methods. Data for HDL binding represents total cell-associated radioactivity measured in NaOH extracts, while data for LDL binding represents dextran-sulfate releasable counts. In a separate experiment with identical conditions, over 50% of the total cell-associated LDL radioactivity was found to be dextran-sulfate releasable. The inset shows Scatchard plots of total HDL3 binding where B represents ng HDL bound per mg cell protein, and F represents the concentration of HDL in the medium in ng/ml.

that can promote cholesterol efflux from cells, such as those in lipoprotein-deficient serum. In the presence of albumin alone, which does not promote net transport of cholesterol from cells, HDL binding activity remained elevated. It is possible that other components of the lipoprotein-deficient serum, such as growth factors, contributed to the reduction in HDL binding activity.

Binding of HDL to AcLDL-treated endothelial cells was a reversible process. When 125I-HDL3 was bound to cells at 4°C, over 70% of the bound protein was released from cells when they were warmed to 37°C (Figure 6). This release was nearly complete within 30 minutes. Most of the released material was insoluble in trichloroacetic acid, indicating that HDL was not degraded substantially by the cells. When cells were maintained at 4°C, almost 50% of the bound HDL was released from cells after the addition of fresh medium. The rate of release at 4°C was less than one-half of that at 37°C, indicating that the release process was temperature-dependent.

Treatment of endothelial cells with AcLDL enhanced the high-affinity, saturable binding of HDL to the cells (Figure 7). Scatchard analysis revealed that this enhanced binding was due to an increase in the maximum binding capacity of the cells, indicative of an increase in the number of binding sites on the
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Figure 8. Competition for 125I-HDL₃ binding at 4°C to endothelial cells by HDL₃, LDL, and AcLDL. Cells were incubated with medium A containing 50 μg/ml AcLDL. After 48 hours, cells were washed, chilled to 4°C, and incubated with medium containing 2 μg/ml 125I-HDL₃ plus the indicated protein concentration of unlabeled HDL₃ (■), LDL (○), or AcLDL (△). After 2 hours, binding was measured as described in Methods. Each value represents the mean of two (no SEM bars) or four to five (plus SEM bars) incubations from two separate experiments.

Results from the present study confirm our previous results showing that the delivery of cholesterol to cells via a receptor-mediated process leads to an increase in cellular cholesterol content, an enhanced rate of cholesterol ester formation, and a suppressed rate of cholesterol synthesis. Thus, HDL binding activity appeared to be regulated in concert with the regulation of other biochemical processes involved in cell cholesterol metabolism, presumably in response to cholesterol loading of cells. The HDL binding sites on bovine aortic endothelial cells have features similar to those described for human fibroblasts and human arterial smooth muscle cells. In all cell types, treatment with sterol-rich medium led to an increase in the number of high-affinity HDL binding sites on the cell surface. Kinetic analysis of the binding data for endothelial cells revealed an apparent Kd of ~3 μg/ml HDL protein, similar to the value of ~2 μg/ml previously reported for fibroblasts. In all cell types, cycloheximide could block the sterol-mediated enhancement of HDL binding activity, suggesting that synthesis of protein is involved in the regulatory process. With both fibroblasts and endothelial cells, most of the HDL bound at 4°C was rapidly released into the medium when cells were subsequently warmed to 37°C. Very little of the bound HDL was directed to lysosomes for degradation. Taken together, these results suggest that these markedly different cell types possess receptors for HDL that have similar properties.

The physical and chemical properties of the HDL binding site are currently unknown. Results showing inhibition of HDL binding activity by cycloheximide treatment have raised the possibility that the binding site may be a protein receptor. In a recent report, Tabas and Tall suggested that HDL binding to cultured fibroblasts and endothelial cells is due to the interaction of HDL particles with membrane lipids, particularly cholesterol, rather than with specific receptors. They based their conclusion on results showing lack of saturability of HDL binding, insensitivity of binding to mild proteolytic degradation, and an apparent direct correlation between membrane cholesterol content and HDL binding activity. They postulated that cycloheximide treatment blocks cholesterol-mediated upregulation of HDL binding to fibroblasts because it decreases the membrane cholesterol content. However, their binding saturation studies were performed under conditions where the cell cholesterol content was low and the expression of receptor activity was expected to be minimal. As demonstrated in the current and previous studies, Scatchard analysis of saturation curves for binding of HDL to cholesterol-loaded cells revealed an apparent single class of high-affinity binding sites for HDL. Moreover, cycloheximide treatment of cholesterol-loaded cells rapidly reversed HDL binding activity under conditions where cell cholesterol content was unchanged (unpublished observations). Clearly, the data available at this time are insufficient to draw definitive conclusions about the molecular nature of the HDL binding site.

Discussion

Results from the present study confirm our previous results showing that the delivery of cholesterol to cells via a receptor-mediated process leads to an increase in HDL binding to the cell surface. Our current results extend these observations to an additional type of delivery process, the "scavenger" receptor pathway. The treatment of bovine aortic endothelial cells with AcLDL (chemically modified LDL that interacts with scavenger receptors but not with native LDL receptors) resulted in a dose-dependent increase in the cells' HDL binding activity. In contrast, AcLDL had little effect on the HDL binding activity of cells that lack scavenger receptors, such as human fibroblasts. Up-regulation of HDL binding to endothelial cells by AcLDL was associated with an increase in cellular cholesterol content, an enhanced rate of cholesterol ester formation, and a suppressed rate of cholesterol synthesis. Thus, HDL binding activity appeared to be regulated in concert with the regulation of other biochemical processes involved in cell cholesterol metabolism, presumably in response to cholesterol loading of cells.
Competition binding studies suggested that the HDL binding sites on endothelial cells have broad specificity; they can interact with different types of lipoproteins. In the presence of increasing concentrations of lipoprotein, both LDL and AcLDL were able to block nearly 50% of the binding of radiolabeled HDL to endothelial cells, although neither of these lipoproteins were as effective as HDL in the maximum ability to block binding. Previous studies\textsuperscript{10} have also shown the partial ability of LDL to compete for HDL binding to cholesterol-treated fibroblasts. Direct binding assays, however, revealed that LDL bound to endothelial cells to a much smaller extent than did HDL. When HDL binding was up-regulated by treatment of cells with AcLDL, LDL binding actually decreased slightly. In the up-regulated state, the molar ratio of cell-associated LDL to HDL was greater than 50 to 1 at similar molar concentrations (see Figure 7). Thus, it is unlikely that the blocking effect that LDL had on HDL binding was strictly due to competition for the same binding sites. It is possible that surface binding of a small quantity of LDL or AcLDL causes a partial inhibition of HDL binding. Alternatively, LDL and AcLDL may interfere with HDL binding through some mechanism that involves a direct interaction between the lipoprotein particles. Havekes et al.\textsuperscript{5} recently reported that direct competition assays using apoprotein-phospholipid vesicles are unreliable because the interaction of vesicles with HDL promotes redistribution of radiolabeled HDL proteins among the competitor particles. Based on the direct binding assay and because of the possible unreliability of the competition assay, we conclude that LDL does not bind to a significant extent to the HDL binding site on endothelial cells.

Recent attention has been focused on the possibility that "foam" cells in the developing atherosclerotic lesion may derive their cholesterol from lipoproteins taken up by cells via the scavenger receptor pathway\textsuperscript{17,13} in tissue culture, cells that possess scavenger receptors can accumulate massive amounts of cholesterol when treated with modified lipoproteins, such as AcLDL.\textsuperscript{13,24} Exposure of these cells to HDL depletes them of this excess cholesterol.\textsuperscript{24,26} It has been postulated\textsuperscript{10,26} that HDL may promote transport of cholesterol from extrahepatic cells by a receptor-mediated process that serves to protect cells from overaccumulation of cholesterol. Results from the present study indicate that HDL binding to cells can be up-regulated when cells are loaded with cholesterol via the scavenger receptor pathway, suggesting that the HDL receptor may play an important role in the prevention of foam cell formation and atherogenesis.

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