Binding of High Density Lipoproteins to Cell Receptors Promotes Translocation of Cholesterol from Intracellular Membranes to the Cell Surface*  

(Received for publication, February 2, 1987)  

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Cultured cells have on their cell surface a specific high-affinity binding site (receptor) for high density lipoproteins (HDL) which appears to promote cholesterol efflux. In this study we characterized the cellular mechanisms involved in HDL receptor-mediated transport of cholesterol from cultured human fibroblasts and bovine aortic endothelial cells. HDL₃, chemically modified by tetranitromethane (TNM-HDL₃), is not recognized by this receptor and was used as a control for efflux not mediated by HDL receptor binding. HDL₃ and TNM-HDL₃ were found to be equally effective in causing efflux of plasma membrane cholesterol radiolabeled with [³H]cholesterol. However, HDL₃ was much more effective than TNM-HDL₃ in causing efflux of [³H]cholesterol associated with intracellular membranes. By measuring movement of endogenously synthesized [³H]cholesterol to the plasma membrane, and into the medium, we found that HDL₃ induced a rapid movement of [³H]cholesterol from a preplasma membrane compartment to the plasma membrane that preceded [³H]cholesterol efflux. This effect was not observed with TNM-HDL₃. Thus, receptor binding of HDL₃ appears to facilitate removal of cellular cholesterol from specific intracellular pools by initiation of translocation of intracellular cholesterol to the plasma membrane.

Human high density lipoproteins (HDL) have the ability to promote cholesterol efflux from many different types of cultured extrahepatic cells, including fibroblasts (1-5), arterial smooth muscle cells (1, 6), and mouse peritoneal macrophages (7, 8). These results are consistent with the hypothesis that HDL serves to transport cholesterol from extrahepatic cells to the liver for ultimate excretion in the bile (9). Studies from this laboratory have shown that several different extrahepatic cell types have receptors on their surface that bind HDL with high affinity and specificity (10-12). This receptor binding of HDL may facilitate the movement of free cholesterol from cells to HDL particles. The purpose of this study was to characterize the cellular mechanism involved in HDL receptor-mediated transport of cholesterol from cells.

EXPERIMENTAL PROCEDURES  

Cells—Bovine aortic endothelial cells were obtained and cultivated as described previously by Schwartz (13). Human skin fibroblasts were grown as previously described (10, 11), using 10% fetal calf serum in Dulbecco's minimum essential medium (GIBCO). Cells were seeded in 35-mm dishes at a density of approximately 150,000 (endothelial cells) or 70,000 (fibroblasts) cells/dish. After 5-6 days, when confluency of fibroblasts was 80%, cells were incubated once with phosphate-buffered saline (PBS, pH 7.4) and were then incubated for 48 h with serum-free minimum essential medium (SF-MEM) containing 2 mg/ml fatty acid free albumin (FAFA) and 50 μg/ml cholesterol (dissolved in ethanol) to upregulate HDL₃ binding (11). The endothelial cells were incubated similarly, except that the incubation medium contained no FAFA and 30 μg/ml acetylated LDL (instead of cholesterol). 

Lipoproteins—Low density (LDL, d = 1.019-1.063 g/ml) and high density (HDL₃, d = 1.125-1.21 g/ml) lipoproteins were isolated by sequential ultracentrifugation technique. HDL₃ was iodinated with ¹²⁵I by the McFarlane monochloride procedure as modified for lipoproteins (13) to a specific activity of approximately 100-250 cpm/ng of protein. Unlabeled HDL₃ was subjected to heparin-agarose affinity chromatography to remove apoB and apoE (10).

Modification of Lipoproteins—LDL was acetylated according to the method of Franek-Conrat (14) as modified by Basu et al. (15). HDL₃ was treated with tetranitromethane (TNM) as described previously (14). Briefly, 20 μl of a freshly prepared solution of 0.6 mM TNM in absolute ethanol was added to 2 ml of HDL₃ (2 mg/ml in 0.9% NaCl; final TNM concentration 6 mM) and the reaction was allowed to proceed in the dark at room temperature for 60 min. The sample was chilled on ice and was subjected to extensive dialysis against 0.9% NaCl with 1 mM EDTA. The resulting TNM-HDL₃ was passed through a 0.22-μm sterile filter and was kept at 4 °C in the dark.

HDL Binding—Binding of HDL₃ to cultured fibroblasts was determined at 4°C as described previously (11). Prior to the binding experiments, cells were loaded with non-lipoprotein cholesterol for 48 h as described earlier. The cells were chilled on ice and washed five times with cold PBS. Then 1.0 ml of cold SF-MEM with 10 mM HEPES, 1 mg/ml FAFA, and 2 μg/ml ¹²⁵I-HDL₃ was added to each dish. For competition studies, binding of ¹²⁵I-HDL₃ to cells was determined in the presence of a 20-fold excess of either HDL₃ or TNM-HDL₃. After 2 h at 0°C the cells were washed three times with cold PBS containing albumin and two times with PBS. Cells were then digested in 0.1 M NaOH and an aliquot was assayed for ¹²⁵I radioactivity and another aliquot was assayed for protein content.

Labeling of Endogenously Synthesized Cholesterol—To enrich the endogenous cholesterol pool with [³H]cholesterol and to minimize its movement to the cell surface, cells were pulsed with [³H]mevalonolactone at 15°C. At this temperature, cells synthesize [³H]cholesterol, albeit slowly, whereas the transfer of newly synthesized [³H]cholesterol to the plasma membrane is markedly reduced (18). [³H]Mevalonolactone was used as a precursor of cholesterol because it bypasses the hydroxymethylglutaryl-CoA reductase step in cholesterol biosynthesis, the activity of which is suppressed in cholesterol-loaded cells. Cholesterol-loaded cells were chilled and washed five times with 2 ml of cold PBS. After the final wash, 1.0 ml of SF-MEM with 10 mM HEPES, 1 mg/ml FAFA, and 0.4 mM [³H]mevalonolactone (10 μCi/μl, New England Nuclear) was added to the cells. The cells were then incubated in a 15 ± 1°C water bath for 6 h. After this pulse, the cells were chilled, washed five times on ice with cold PBS, and used for measurement of [³H]cholesterol efflux.
Receptor-mediated Cholesterol Efflux

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was not necessary for efflux of exogenously labeled plasma membrane cholesterol. Although efflux of plasma membrane-derived \(^{[3]H}\)cholesterol appeared to be saturable with respect to the acceptor concentration, the apparent saturability may have been due to tracer depletion from the membrane pool rather than saturation of membrane binding sites, since more than 50% of the total cellular \(^{[3]H}\)cholesterol was removed from cells during the 4-h experiment.

To test whether the interaction between HDL and the cell-surface receptor leads to an enhanced efflux of newly synthesized \(^{[3]H}\)cholesterol not yet in the plasma membrane cholesterol pool, cholesterol-loaded cells were incubated with \(^{[3]H}\)mevalonolactone at a low temperature (15 °C) for 6 h. At this temperature the movement of endogenously synthesized \(^{[3]H}\)cholesterol to the plasma membrane is minimized, thus allowing a specific enrichment of the intracellular cholesterol pool with \(^{[3]H}\)cholesterol prior to the efflux incubation. After a typical pulse labeling at 15 °C, less than 10% of the total cellular \(^{3}H\) label was located in the plasma membrane (as measured by conversion of \(^{[3]H}\)cholesterol to \(^{[3]H}\)cholestenone after treatment of cells with cholesterol oxidase). In contrast, when the plasma membrane was labeled with an exogenous source of \(^{[3]H}\)cholesterol, more than 75% of the total cell label was oxidized by this treatment (data not shown).

After the pulse incubation at 15 °C with \(^{[3]H}\)mevalonolactone, the cells were chased with unlabelled mevalonolactone (0.4 mM) at 37 °C in the presence of different amounts of either HDL or TNM-HDL. With human skin fibroblasts, addition of HDL to the medium caused a marked increase in efflux of endogenously synthesized \(^{[3]H}\)cholesterol during a 2-h chase incubation (Fig. 2A). At 100 μg/ml of HDL, more than 20% (total minus control) of the intracellular \(^{3}H\) label had been removed from the cells. At the same concentrations, TNM-HDL was much less effective than HDL in promotion of cholesterol efflux. When efflux of endogenously synthesized \(^{[3]H}\)cholesterol was measured in endothelial cells using the same protocol, a similar efflux pattern could be observed (Fig. 2B). During a 1-h chase incubation, HDL was again much more effective in promoting \(^{[3]H}\)cholesterol efflux compared to TNM-HDL. In both cells types, the efflux of endogenously synthesized \(^{[3]H}\)cholesterol from HDL was characterized by high-affinity and low-affinity components. The efflux pattern

FIG. 1. Efflux of plasma membrane \(^{[3]H}\)cholesterol from fibroblasts to HDL and TNM-HDL. Cholesterol-loaded fibroblasts were pulse-labeled with 0.2 μCi/ml \(^{[3]H}\)cholesterol (55 Ci/mmoll, added in ethanol) for 6 h at 15 °C. Efflux of \(^{[3]H}\)cholesterol was measured at 37 °C in the presence of the indicated protein concentrations of either HDL (■) or TNM-HDL (○) during a 4-h incubation, as described under "Experimental Procedures." Results represent the mean ± S.D. of values for medium \(^{[3]H}\)cholesterol from four incubations, expressed as percent of the initial cellular \(^{[3]H}\)cholesterol.

Results

Previous reports from this laboratory have shown that tetranimethane-modified HDL particles (TNM-HDL) lose their ability to compete with \(^{125}I\)-HDL for binding to the HDL receptor on fibroblasts and endothelial cells (14). Therefore, TNM-HDL was used in this study as a negative control for HDL to assess the effects of the receptor/HDL interaction on cellular cholesterol efflux. Before using the TNM-HDL preparations, we routinely examined the ability of these modified HDL particles to compete with \(^{125}I\)-HDL for receptor binding. Typically, TNM-HDL at a 20-fold excess displaced less than 15% of \(^{125}I\)-HDL from the high-affinity binding sites, whereas the same excess of HDL displaced more than 80% of the bound \(^{125}I\)-HDL (data not shown, cf. Ref. 14).

To examine the ability of HDL and TNM-HDL to cause efflux of plasma membrane cholesterol radiolabeled from an exogenous source, cells were pulsed with \(^{[3]H}\)cholesterol (added in ethanol) and efflux was measured during a 4-h incubation at 37 °C. Over the 4-h efflux period, both HDL and TNM-HDL at concentrations between 5 and 100 μg of protein/ml were equally effective in removing \(^{[3]H}\)cholesterol from cells (Fig. 1), indicating that receptor binding of HDL
mediated by TNM-HDL<sub>3</sub> appeared to be characterized mainly by a low-affinity component, since little saturability could be observed (Fig. 2, A and B).

To test whether the receptor/HDL<sub>3</sub> interaction in some way promotes the movement of intracellular [<sup>3</sup>H]cholesterol to the cell surface, the appearance of endogenously synthesized [<sup>3</sup>H]cholesterol in the plasma membrane was measured. This was done using cholesterol oxidase as an extracellular probe for plasma membrane cholesterol. Since this enzyme has access only to cholesterol on the cell surface, it is subsequently released into the medium.

The results indicate that HDL<sub>3</sub>, but not TNM-HDL<sub>3</sub>, promotes movement of intracellular [<sup>3</sup>H]cholesterol to the cell surface. HDL<sub>3</sub> at 20 mg/ml markedly enhanced the movement of intracellular [<sup>3</sup>H]cholesterol to the cell surface (Fig. 3B). This movement was faster in endothelial cells than in fibroblasts. The movement of newly synthesized [<sup>3</sup>H]cholesterol to the cell surface in cells incubated with TNM-HDL<sub>3</sub> was similar to that observed in cells incubated with lipoprotein-free medium. A comparison of the appearance of the [<sup>3</sup>H]cholesterol in the plasma membrane and in the incubation medium showed that [<sup>3</sup>H]cholesterol first appeared on the cell surface but soon thereafter was found in the medium (Fig. 3C). These results indicate that HDL<sub>3</sub>, but not TNM-HDL<sub>3</sub>, promotes movement of cholesterol from intracellular membranes to the cell surface, where it is subsequently released into the medium.

Receptor binding of HDL<sub>3</sub> appears to specifically promote efflux of endogenously labeled cholesterol that resides within intracellular membranes. When incubations with [<sup>3</sup>H]mevalonolactone were extended to 25 h at 37 °C so that most of the [<sup>3</sup>H]cholesterol label was in the plasma membrane (as determined by cholesterol oxidase), HDL<sub>3</sub> promoted [<sup>3</sup>H]cholesterol efflux by a nonsaturable, low-affinity process that was identical to that observed with TNM-HDL<sub>3</sub> (Fig. 4). This finding is consistent with the efflux pattern shown in Fig. 1, where the plasma membrane cholesterol pool was labeled exogenously with [<sup>3</sup>H]cholesterol. Thus, removal of cholesterol from plasma membranes by HDL does not appear to be receptor-mediated, regardless of whether the plasma membrane cholesterol is derived from exogenous or endogenous sources.

**Discussion**

Results in the present study show that the interaction between HDL<sub>3</sub> and its cell-surface receptor on cultured extrahepatic cells promotes selective movement of intracellular membrane-associated [<sup>3</sup>H]cholesterol to the plasma membrane and subsequent efflux of the translocated cholesterol.
This succession of events was not seen in cells incubated with TNM-modified HDL₃, which does not bind to the HDL receptor (14).

The observed HDL₃-mediated [³H]cholesterol efflux appeared to be characterized by two different processes. First, as a result of receptor/HDL₃ interaction, [³H]cholesterol was rapidly translocated from a preplasma membrane compartment to the plasma membrane. The second process was the actual efflux or removal of [³H]cholesterol from cells to the acceptor particles. Results suggest that the receptor/HDL₃ interaction was necessary only to trigger the initial translocation process. Once the [³H]cholesterol was in the plasma membrane, both HDL₃ and TNM-HDL₃ appeared to be equally effective in removing [³H]cholesterol from cells. This conclusion was based on studies showing that efflux of [³H]cholesterol already in the plasma membrane cholesterol pool was identical in the presence of both forms of HDL₃. The lack of involvement of receptor binding in removal of cholesterol from plasma membranes is not surprising, since this process is thought to be mediated by spontaneous surface transfer (22). The endogenous [³H]cholesterol that was translocated and removed from cells following receptor binding of HDL₃ appears to be derived from the putative intracellular regulatory cholesterol pool, since fibroblasts incubated with HDL₃ show a significant and rapid down-regulation of the activity of the intracellular cholesterol esterifying enzyme, acyl-CoA:cholesterol acyltransferase activity (5, 14). TNM-HDL₃, which neither binds to the HDL receptor nor stimulates cholesterol translocation, also does not down-regulate the activity of acyl-CoA:cholesterol acyltransferase (14).

In the absence of HDL, the movement of endogenously synthesized [³H]cholesterol to the cellular plasma membrane was a slow process in cholesterol-loaded cells. This is in contrast to results obtained for cholesterol movement in cholesterol-depleted cells (23). It is possible that, with cholesterol-loaded cells, endogenously synthesized cholesterol is readily equilibrated with intracellular pools of excess free cholesterol, which are only partially translocated to the cell surface at a slow rate. Although HDL₃ accelerated movement of intracellular [³H]cholesterol to the cell surface in both fibroblasts and endothelial cells, the amount of [³H]cholesterol that was translocated and the kinetics of the processes were different in these two cell types, with endothelial cells translocating more cholesterol at a faster rate than fibroblasts. Despite these differences in rates of translocation, the rate of [³H]cholesterol efflux was similar in both cell types, suggesting that the efflux process per se may be rate-limiting.

Results in the present study do not allow conclusions to be drawn as to the mechanism of the HDL₃-induced translocation of intracellular cholesterol to the cell surface. It has been proposed that, at least in macrophages, receptor binding of HDL can lead to endocytosis and resurfacing of receptor-HDL complexes, thus providing a targeting mechanism whereby HDL complexes can remove cholesterol from specific intracellular compartments (24). However, this mechanism is an unlikely explanation for the effect of HDL₃ on cholesterol translocation since it has been shown that, under incubation conditions similar to those used in this study, internalization of HDL₃ could not be detected (25).

Thus, the results of this study suggest the existence of a specific biological response to the interaction between HDL₃ and its receptor. It is known that HDL₃ receptor activity is a function of total cellular unesterified cholesterol mass (11, 12). These results suggest that the function of binding of HDL₃ to its receptor is to specifically promote transport of excess intracellular cholesterol to the cell surface where it can be removed from cells by HDL or other acceptor particles.

Acknowledgments—We thank Maria Culala, Thomas Johnson, and Karin Sundquist for skillful technical assistance.

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