0014-4754/93/490561-06$1.50 + 0.20

Net transport of cholesterol from cells of the human EA.hy 926 endothelial cell line to high density lipoproteins

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Received 29 June 1992; accepted 22 February 1993

Abstract. EA.hy 926 cells, a human endothelial cell line, show characteristics of differentiated endothelial cells. The cells express saturable binding of apo E-free ^{125}I-high density lipoprotein$_3$ (HDL_3). B_{max} increased from 71 to 226 ng HDL_3 bound/mg cell protein after cholesterol loading of the confluent endothelial cells with cationized low density lipoprotein (LDL). The affinity did not change after cholesterol enrichment (K_d was 37 μg HDL_3 protein/ml for control cells and 31 μg/ml for loaded cells). Incubation of cholesterol-loaded EA.hy 926 cells with native HDL and LDL had different effects on cellular cholesterol levels. Incubation with HDL decreased both esterified and unesterified cellular cholesterol, but LDL did not change total cellular cholesterol. However, LDL tended to increase cellular cholesteryl esters, with a concomitant decrease of unesterified cellular cholesterol. Incubation of endothelial cells with both HDL and LDL also resulted in decreased total cellular cholesterol levels. These data show that cationized LDL-loaded human endothelial EA.hy 926 cells can be used to study the net transport of cellular cholesterol to HDL, the first step in reverse cholesterol transport.
Key words. Binding; cholesterol transport; high density lipoprotein; low density lipoprotein.

High density lipoprotein (HDL) is believed to play a role in the transfer of peripheral cholesterol back to the liver, a process called reverse cholesterol transport. HDL-binding membrane proteins have been visualized in different types of cells[1-5], but the physiological role of these HDL-binding proteins is still unclear. For various cell types, including bovine vascular endothelial cells, up-regulation of HDL-binding activity after enrichment of the cells with cholesterol has been reported[6-10]. This suggests a functional role for HDL-binding proteins in the removal of excess cellular cholesterol. However, there are also investigators who deny that HDL binds to membrane proteins[11,12] or that HDL binding plays a role in cholesterol efflux[13-15].

In vivo, vascular endothelial cells are in contact with high concentrations of plasma lipoproteins. The endothelial cell monolayer is the primary site of interaction between the vessel wall, blood cells and plasma lipoproteins. It exerts a protective role against thrombosis and atherogenesis. Lipoprotein uptake by vascular endothelial cells has been implicated in the initiation and development of atherosclerosis[16,17]. In vivo, cholesterol uptake by endothelial cells can take place via several mechanisms. As it can be expected that low density lipoprotein (LDL)-receptors are down-regulated in vivo, receptor-mediated endocytosis of apo B or apo E-containing lipoproteins is unlikely. It has been reported, however, that endothelial cells can take up LDL via non-specific endocytosis or pinocytosis[18]. A second possibility is the uptake of cholesterol from chylomicrons, as suggested by Fielding et al.[19].

The first step of reverse cholesterol transport, egress of cholesterol from endothelial cells, can be studied in vitro. EA.hy 926 cells, a human endothelial cell line, show characteristics of differentiated endothelial cells, e.g. expression of von Willebrand factor[20], tissue plasminogen activator[21], plasminogen activator inhibitor-type 1[21] and production of prostacyclin[22]. These cells still express typical differential characteristics after more than one hundred cumulative population doublings[20,21]. We studied net mass transport of cholesterol from these endothelial cells after enrichment of the cells with intracellular cholesterol derived from cationized LDL. Although the effects of cholesterol enrichment on binding of HDL_3 to endothelial cells have been studied before[6,7], very little is known about the egress of cholesterol from endothelial cells. In this study we examined 1) the effect of cell cholesterol enrichment on the binding of apo E-free HDL_3 to EA.hy 926 cells, 2) whether net mass transport of cholesterol from EA.hy 926 to HDL is dependent on the cellular concentration of cholesterol, and 3) whether incubation with LDL or LDL plus HDL is also able to release cholesterol from loaded EA.hy 926 cells.

Our results indicate that EA.hy 926 cells are suitable for further studies on the mechanism of net mass transport of cellular cholesterol to HDL.

Materials and methods

Cell culture. The endothelial cell line EA.hy 926 was generously provided by Dr. C.-J. S. Edgell[20]. EA.hy 926 cells were cultured in DMEM, supplemented with 10%

FCS, 2 mM L-glutamine, 100 U penicillin/ml, 100 µg streptomycin/ml and HAT (100 µM hypoxanthine Merck No. 4517; 0.4 µM aminopterin, Serva No. 13170; 16 µM thymidine, Merck No. 8206) at 37 °C in 5% CO_2 and 95% air. Cells were plated in multiwell dishes (22 mm: $0.5 \cdot 10^4$ cells/well, 35 mm: $1 \cdot 10^5$ cells/well) 4 to 5 d prior to each experiment. Medium was changed every 2 or 3 d. The confluent cells were loaded with cholesterol during a 24 h incubation of the cells with cationized LDL in growth medium as described earlier[23]. Control cells were preincubated similarly with growth medium (containing 10% FCS) only.

Lipoproteins. HDL (density range 1.063–1.21 g/ml), HDL_3 (density range 1.125–1.21 g/ml) and LDL (density range 1.006–1.063) were isolated by sequential ultracentrifugation of human plasma according to Havel et al.[24]. Apo E-free HDL_3 was isolated by chromatography of HDL_3 on a heparin-Sepharose column[25]. Apo E-free HDL_3 was iodinated using the iodine monochloride method (using $Na^{125}I$ obtained from Amersham International) as described earlier[26]. The molar iodine-to-protein ratio of the ^{125}I-HDL_3 preparations ranged from 0.22 to 0.49; 95.4 ± 0.8% of the label was protein-bound, 1.7 ± 0.6% was in the HDL-lipids and 2.9 ± 0.5% was free. All lipoproteins were dialyzed against 0.9% NaCl with 10 mM sodium phosphate (pH 7.4) and 1 mM EDTA. Before cell experiments, the lipoproteins were dialyzed for a second time against DMEM and filter sterilized (0.45 µm).

LDL was cationized according to Basu et al.[27]. An equal volume of LDL (20 to 30 mg of protein) was added to a 2 M solution of 3-dimethylaminopropylamine (Aldrich No. 24.005-2) in H_2O at room temperature. The pH was adjusted to 6.5 with HCl. After stirring this mixture, 100 mg of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (Sigma No. E-7750) was added. The pH was maintained at 6.5 with 0.1 M HCl during the reaction. After 3 hours, the pH of the solution did not change any more. The reaction mixture was left at 4 °C overnight. The cationized LDL was dialyzed against 0.9% NaCl with 10 mM sodium phosphate (pH 7.4) and 1 mM EDTA, filter sterilized (0.8 µm) and stored. Storage of cationized LDL at +4 or −20 °C did not influence the uptake by EA.hy 926 cells.

Binding of ^{125}I-HDL_3 to EA.hy 926 cells. Confluent EA.hy 926 cells (in 22 mm wells) were preincubated with 50 µg catLDL-TC/ml in growth medium for 24 h (0.5 ml/well). Control cells were preincubated similarly with growth medium only. After cholesterol enrichment, the wells were rinsed three times with DMEM containing 2 mg BSA/ml (Sigma No. A-4378) and penicillin/streptomycin at room temperature, followed by three rinses with cold DMEM containing only penicillin/streptomycin. Subsequently, media were added (DMEM, supplemented with 2 mg BSA/ml, L-glutamine, penicillin/streptomycin and lipoproteins as indicated, 0.5 ml/well) and the cells were incubated for 4 h at 4 °C in an atmosphere with 5% CO_2. After the incubations, the cells were rinsed three times with 2 mg BSA/ml in 0.9% NaCl/50 mM Tris-HCL (pH 7.4) and three times with 0.9% NaCl/50 mM Tris-HCl (pH 7.4). Cell protein was dissolved in 300 µl 1 M NaOH. The radioactivity in this solution was counted using a Packard Minaxi 5000 gamma counter and protein was measured[28] in the same aliquot.

Saturable binding of apo E-free ^{125}I-HDL_3 to control cells (after preincubation with growth medium only) and cholesterol-loaded cells (after preincubation with growth medium plus cationized LDL) was measured at 5–100 µg ^{125}I-HDL_3 protein/ml (specific activity 200 cpm/ng). Non-specific binding was measured in the presence of 1 mg unlabelled HDL_3/ml. Specific binding was calculated by difference of total and non-specific binding.

Measurement of cholesterol transport from EA.hy 926 cells to HDL. After loading with cationized LDL the cells (in 35 mm wells) were rinsed twice with DMEM, containing 2 mg BSA/ml and penicillin/streptomycin, and twice with DMEM plus penicillin/streptomycin only, at room temperature. The cells were subsequently incubated for 24 h at 37 °C with 1 ml efflux medium in an atmosphere with 5% CO_2 and 95% air. Media consisted of DMEM, supplemented with L-glutamine, penicillin/streptomycin and 2 mg BSA/ml (Sigma A-4378) and lipoproteins as indicated. Experiments were ended by cooling the culture dishes on ice. The culture media were collected and small amounts of dislodged cells were spun down ($500 \times g_{max}$, 4 °C). Cell media were stored at −20 °C until cholesterol was determined. The wells were rinsed three times in 0.9% NaCl with 2 mg BSA/ml and 50 mM Tris-HCl (pH 7.4), followed by three rinses with 0.9% NaCl/Tris-HCl (50 mM, pH 7.4). The cells were scraped into 1 ml 0.9% NaCl/Tris-HCl (50 mM, pH 7.4) with a rubber policeman and lipids were extracted using the method of Bligh and Dyer[29]. The resulting protein pellet was dissolved in 500 µl 0.1 M NaOH containing 10% SDS (w/v). Cell protein was measured according to Lowry et al.[28].

Cellular lipids were dried down under nitrogen and dissolved in 300 µl 2-propanol. The lipid extracts were stored up to 1 day at −20 °C, and unesterified and total cholesterol were determined separately using a slight modification of the method described by Heider and Boyett[30]. For determination of unesterified cholesterol the reaction mixture consisted of 0.15 mg p-OH-phenylacetic acid/ml (Sigma No. H-4377), 5 U peroxidase/ml (Boehringer Mannheim No. 108081) and 3.2 µg cholesterol oxidase/ml (Boehringer Mannheim No. 396818) in sodium phosphate buffer (50 mM, pH 7.4). The reaction mixture for the total cholesterol determination was as for unesterified cholesterol, plus 6.4 µg cholesteryl esterase/ml (Boehringer Mannheim No. 161772), 5 mM

taurocholate (Calbiochem No. 580217) and 0.17 mM PEG-6000 (Serva No. 33137). 0.75 ml of reaction mixture was added to 40 µl cell extract in 2-propanol. A 100 µg/ml solution of cholesterol (Sigma No. C-8253) in 2-propanol was used as a standard. After the incubation (37 °C, 30 min for unesterified cholesterol and 120 min for total cholesterol assays) the reaction was ended by addition of 1.5 ml 0.5 M NaOH and fluorescence was measured with a Perkin-Elmer LS-3B fluorimeter (excitation 325 nm, emission 415 nm). Total and unesterified cholesterol in the incubation media were measured using a commercially available kit (cholesterol kit No. 310328, cholesterol esterase No. 161772, Boehringer Mannheim).

Statistical analyses. The non-parametric Mann-Whitney test was used for comparison between treatment groups with unpaired observations. For paired observations the non-parametric paired Wilcoxon test was used. The differences were considered to be statistically significant if $p < 0.05$.

Results

Figure 1 shows the binding of apo E-free ^{125}I-HDL_3 to control and cholesterol-loaded EA.hy 926 cells. Total as well as specific binding was higher for the cholesterol-enriched cells. Table 1 shows the K_d and B_{max} of binding of apo E-free HDL_3 to control and cholesterol-loaded EA.hy 926 cells. Cholesterol enrichment did not change the affinity for HDL_3 (K_d was about 37 and 31 µg/ml, respectively), but the maximal binding increased from about 71 to 226 ng HDL_3/mg cell protein for control and cholesterol-loaded cells.

Table 2 shows that after 24 h of incubation of EA.hy 926 cells with 50 µg of catLDL-TC/ml, total cellular cholesterol increased about 4 fold. Both unesterified and esterified cholesterol increased after loading with catLDL. Subsequent incubation of cholesterol-enriched cells with HDL decreased cell cholesterol significantly, in comparison to incubation with BSA alone. After incubation with 2 mg of HDL/ml, both cell UC and EC levels decreased.

After loading EA.hy 926 cells with different concentrations of cationized LDL, cholesterol transport was measured after 24 h of incubation with 0.2 mg HDL_3 protein/ml (see fig. 2). After the preincubation with 0,

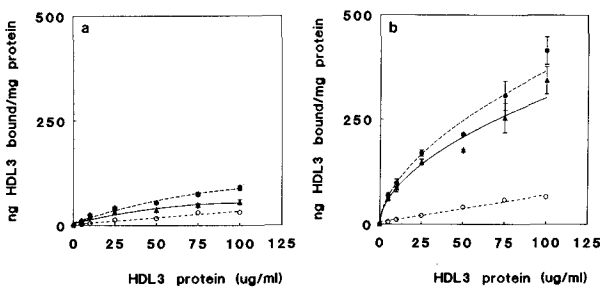

Figure 1. Binding of apo E-free ^{125}I-HDL_3 to a) control (preincubated in medium with 10% FCS) and b) cholesterol-loaded EA.hy 926 cells (preincubated in medium with 10% FCS plus 50 µg catLDL-TC/ml). Total (-●-), specific (-△-) and non-specific (-○-) binding per mg cell protein was measured after 4 h incubation at 4 °C. Non-specific binding was determined after addition of 1 mg unlabelled HDL_3/ml, specific binding was calculated by difference of total and non-specific binding. Values are means ± SEM (n = 3).

Table 1. Binding characteristics of apo E-free HDL_3 to control and cholesterol-loaded EA.hy 926 cells.

	K_d^a	B_{max}^b
Control cells		
Experiment 1	31	62
Experiment 2	42	79
CatLDL-loaded cells[c]	31 ± 4	226 ± 64

[a]Values are in µg HDL_3 protein/ml. [b]Values are in ng HDL_3 protein bound/mg cell protein. [c]Confluent EA.hy 926 cells were incubated with 50 µg catLDL-TC/ml for 24 h at 37 °C. Values are means ± SEM of 4 separate experiments.

Table 2. Loading and net mass transport of cholesterol from EA.hy 926 cells to HDL

	µg Cholesterol/mg cell protein[a]		
	TC	UC	EC
Control cells[b]	46.3 ± 5.0	39.7 ± 5.8	6.6 ± 1.5
Loaded cells	166.4 ± 12.0	96.0 ± 6.2	70.5 ± 11.5
After 24 h of incubation			
0.2% BSA	165.6 ± 0.4	101.5 ± 3.8	64.1 ± 3.9
0.2% BSA, plus HDL	121.7 ± 9.3[c]	76.6 ± 7.8[c]	45.1 ± 3.1[c]

[a]Values are means ± SD (n = 3). [b]Cells were preincubated for 24 h, control cells in medium with 10% fcs and loaded cells in the same medium plus 50 µg cationized LDL-TC/ml. After the preincubation the loaded cells were rinsed, and incubated for 24 h in medium with BSA only or with BSA plus 2 mg HDL-protein/ml. [c]Significantly different from BSA alone (Mann-Whitney, $p < 0.05$).

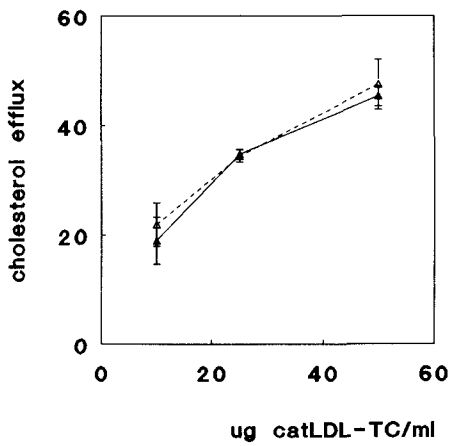

Figure 2. EA.hy 926 cells were loaded with increasing concentrations of catLDL for 24 h at 37 °C. Subsequently, net mass transport of cellular cholesterol to HDL was measured as the increase of cholesterol in the medium after 24 h of incubation with 0.2 mg HDL_3/ml at 37 °C. Medium UC (-▲-) and TC (-△-) were both determined. Cholesterol transport was expressed in nmol cholesterol/mg cell protein (means ± SEM, n = 6).

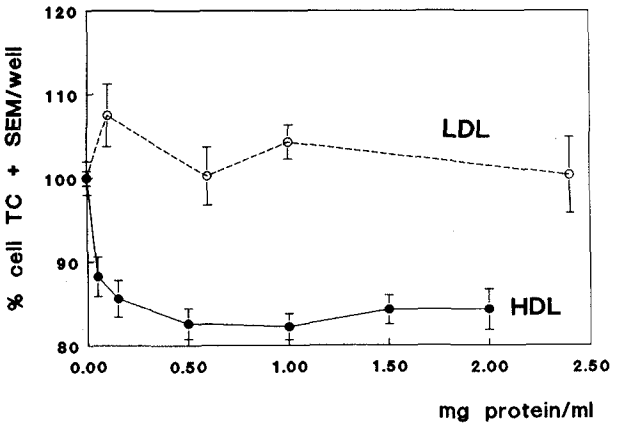

Figure 3. Net mass transport of cell TC from cholesterol-loaded EA.hy 926 cells to HDL (-●-) and LDL (-○-). After 24 h of incubation with DMEM, supplemented with L-glutamine, penicillin/streptomycin, 2 mg BSA/ml and lipoproteins as indicated, cells were washed, scraped into buffer and extracted, and cell TC was measured fluorimetrically as described in '*Materials and methods*'. Values are expressed as % of cell TC after incubation with BSA alone (means ± SEM).

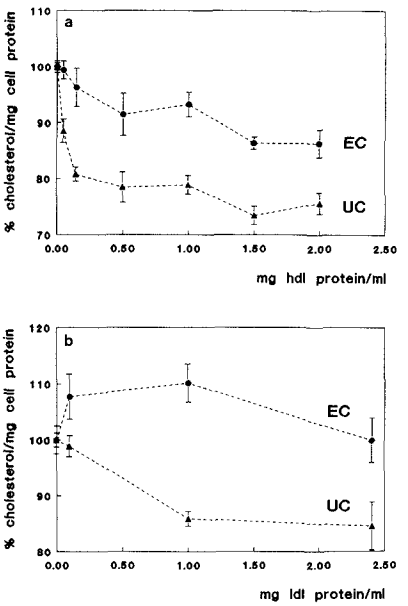

Figure 4. Transport of cell UC (-▲-) and EC (-●-) from cholesterol-loaded EA.hy 926 cells to a) HDL and b) LDL. Cells were incubated and analyzed as in figure 3. Both TC and UC were measured in the cellular lipid extracts, EC was calculated by difference between TC and UC. Values are expressed as % of cell UC and EC after incubation with BSA alone (means ± SEM).

10, 25 and 50 µg catLDL-TC/ml, cellular cholesterol was 34 ± 1, 56 ± 7, 105 ± 6 and 155 ± 3 µg/mg cell protein, respectively (means ± SD, n = 6). The net mass transport of cholesterol, measured as the increase of cholesterol in the medium, was dependent on the loading of the cells (see fig. 2). Under all conditions tested the increase in medium total cholesterol consisted quantitatively of unesterified cholesterol.

Figure 3 shows that net mass transport of cholesterol was specific for HDL, since LDL did not lower cellular cholesterol content. Cholesterol transport to HDL was already maximal at 0.15 mg of HDL protein/ml. All HDL concentrations, ranging from 0.15 to 2.0 mg of HDL protein/ml, reduced total cell cholesterol significantly ($p < 0.01$). At all LDL concentrations used, no significant reduction of total cellular cholesterol by LDL could be detected. Comparable results were obtained if cell cholesterol was expressed as µg cholesterol per mg cell protein (not shown).

The transport of cholesterol to HDL resulted in a decrease of both cell UC and cell EC (see fig. 4a). At low concentrations of HDL, the reduction of cell TC was caused mainly by the release of unesterified cholesterol. Cell UC was significantly decreased at all HDL concentrations used, in comparison with incubation with BSA only ($p < 0.01$, non-parametric Mann-Whitney test). Cell EC was also significantly lowered after incubation with 0.15–2.0 mg HDL/ml ($p < 0.05$). Concentrations of about 0.2 mg HDL/ml and higher resulted in maximal transport of cell cholesterol: cell UC decreased 20–25% and cell EC decreased 10–15%. After incubation with LDL, cell EC tended to increase, with a concomitant decrease in UC (see fig. 4b). The decrease of cell UC reached statistical significance, compared with UC concentration after incubation with BSA only ($p < 0.01$, non-parametric Mann-Whitney test), at concentrations of 1.0–2.4 mg LDL protein/ml. Table 3 shows the cell TC levels after incubation of loaded EA.hy 926 cells with BSA, or physiological concentrations of HDL, LDL and HDL plus LDL. As expected, HDL decreased cell TC and LDL did not. Cell cholesterol also decreased if the cells were incubated with a combination of HDL and LDL. Incubation with HDL alone, or HDL plus LDL, resulted in a significantly lower cell TC levels than incubation with LDL, indicating that LDL did not inhibit net mass transport of cellular cholesterol to HDL.

Table 3. Cellular cholesterol levels after incubation of cholesterol-loaded EA.hy 926 cells with HDL and LDL

Treatment (n)[a]	µg TC/mg cell protein
BSA (2)	145.7 ± 2.5
BSA + HDL (3)	129.1 ± 6.3[b]
BSA + LDL (3)	143.7 ± 2.0
BSA + HDL + LDL (3)	129.2 ± 6.8[b]

[a]After loading the EA.hy 926 cells with cationized LDL the cells were washed and incubated with DMEM, containing L-glutamine, penicillin/streptomycin, 2 mg BSA/ml and lipoproteins as indicated (concentrations used were 2 mg of HDL protein/ml and 1 mg of LDL protein/ml). After 24 h of incubation at 37 °C the cells were washed, scraped into buffer and extracted, and cell TC and protein were determined as described in 'Materials and methods'. Values are means ± SD (n = 2–3). [b]Significantly different from incubation with LDL ($p < 0.05$, non-parametric Mann-Whitney test).

Discussion

Up-regulation of HDL binding after cholesterol enrichment of endothelial cells, e.g. by incubation of the cells with acetylated LDL[6], with 25-hydroxy cholesterol[7] or with unesterified cholesterol[31], has been reported before. The present paper reports that, after cholesterol enrichment with cationized LDL, EA.hy 926 cells also show increased binding (B_{max}) of apo E-free ^{125}I-HDL_3, without there being a significant effect on the dissociation constant (K_d). The K_d for HDL_3 binding to EA.hy 926 cells (31–37 μg/ml) was higher than for bovine vascular endothelial cells (3–10 μg/ml, see Brinton et al.[6] and Tauber et al.[7]). This difference may be caused by the differentiation of the EA.hy 926 cells. This would imply that EA.hy 926 cells do not fully express all characteristics of primary endothelial cells. However, for HDL_3 binding to peripheral cells a K_d of 30–40 μg/ml is a common value. A large variation in the K_d values for HDL binding to various cell types is found in the literature. Values range from 5 to 95 μg/ml[6–10].

Net mass transport of cholesterol was measured in two ways: 1) by measurement of the increased cholesterol concentration in the incubation medium, and 2) by assays of the decrease in cell cholesterol after extraction of the cells. The first method showed similar increases of unesterified and total cholesterol in the medium after 24 h of incubation with HDL_3 (see fig. 2). As cholesteryl esters did not appear in the medium, these data indicate that cholesterol leaves the cells in the unesterified form and that esterification does not occur in the medium during the incubation of the cells with HDL. In separate experiments we could not detect any lecithin:cholesteryl acyltransferase activity in ultracentrifugally isolated HDL, which were used in the experiments (unpublished observation). The lack of increase of medium cholesteryl esters also indicates that no (possibly) extracellularly bound cationized LDL was released from the cells during incubation.

Results on cholesterol transport obtained with the second method (i.e. assay of the decrease of cellular cholesterol, see fig. 3) indicate that transport of cholesterol to HDL was almost maximal, with HDL concentrations as low as 0.15 mg/ml. Apparently, cholesterol transport was limited by factors other than the HDL concentration, e.g. the desorption of cholesterol out of the plasma membrane, the transfer of intracellular cholesterol to the plasma membrane, or the hydrolysis of intracellular cholesteryl esters. The decrease of cellular cholesterol consisted of a decrease of both esterified and unesterified cholesterol (after incubation with HDL, see fig. 4a). LDL also decreased cellular unesterified cholesterol, but cholesteryl esters increased, resulting in virtually unchanged cell TC levels (see fig. 4b). Probably this can be explained by a stimulation of the intracellular cholesterol esterification by LDL. If both HDL and LDL were present together during the experiments, there was a similar decrease in cell TC to that seen with incubation with HDL alone (see table 3). So, net mass transport of cellular cholesterol to HDL is not inhibited by LDL.

To summarize, the EA.hy 926 cells respond normally to cholesterol loading with respect to HDL binding and net mass transport of cellular cholesterol to HDL. Therefore, the cationized LDL-loaded EA.hy 926 cell model can be used to study the relation between HDL binding to specific high affinity binding sites and the net mass transport of cellular cholesterol to HDL.

Abbreviations: apo E, apolipoprotein E; BSA, bovine serum albumine; catLDL, cationized LDL; DMEM, Dulbecco's modified Eagle's medium; EC, esterified cholesterol; HDL, high density lipoprotein; FCS, foetal calf serum; LDL, low density lipoprotein; TC, total cholesterol; UC, unesterified cholesterol.

Acknowledgements. We want to thank Dr. C.-J. S. Edgell for generously providing the EA.hy 926 cells. This research was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organisation for Scientific Research (NWO).

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