The human breast carcinoma cell line HBL-100 acquires exogenous cholesterol from high-density lipoprotein via CLA-1 (CD-36 and LIMPII analogous 1)-mediated selective cholesteryl ester uptake

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Aberrant cell proliferation is one of the hallmarks of carcinogenesis, and cholesterol is thought to play an important role during cell proliferation and cancer progression. In the present study we examined the pathways that could contribute to enhanced proliferation rates of HBL-100 cells in the presence of apolipoprotein E-depleted high-density lipoprotein subclass 3 (HDL_3). When HBL-100 cells were cultivated in the presence of HDL_3 (up to 200 μg/ml HDL_3 protein), the growth rates and cellular cholesterol content were directly related to the concentrations of HDL_3 in the culture medium. In principle, two pathways can contribute to cholesterol/cholesteryl ester (CE) uptake from HDL_3, (i) holoparticle- and (ii) scavenger-receptor BI (SR-BI)-mediated selective uptake of HDL_3-associated CEs. Northern- and Western-blot analyses revealed the expression of CLA-1 (CD-36 and LIMPII analogous 1), the human homologue of the rodent HDL receptor SR-BI. In line with CLA-1 expression, selective uptake of HDL_3-CEs exceeded HDL_3-holoparticle uptake between 12- and 58-fold. Competition experiments demonstrated that CLA-1 ligands (oxidized HDL, oxidized and acetylated low-density lipoprotein and phosphatidylserine) inhibited selective HDL_3-CE uptake. In line with the ligand-binding specificity of CLA-1, phosphatidylcholine did not compete for selective HDL_3-CE uptake. Selective uptake was regulated by the availability of exogenous cholesterol and PMA, but not by adrenocorticotropic hormone. HPLC analysis revealed that a substantial part of HDL_3-CE, which was taken up selectively, was subjected to intracellular hydrolysis. A potential candidate facilitating extralysosomal hydrolysis of HDL_3-CE is hormone-sensitive lipase, an enzyme which was identified in HBL-100 cells by Western blots. Our findings demonstrate that HBL-100 cells are able to acquire HDL-CEs via selective uptake. Subsequent partial hydrolysis by hormone-sensitive lipase could provide 'free' cholesterol that is available for the synthesis of cellular membranes during proliferation of cancer cells.

Key words: cancer cell, cell growth, human scavenger receptor class B type I.

INTRODUCTION

Numerous studies have implicated an important role for cholesterol during proliferation and progression of cancer. In principle, rapidly dividing cancer cells have two major possibilities to fulfil their need for cholesterol. The cellular requirements can be met by either *de novo* biosynthesis or uptake of exogenous lipoprotein-associated cholesterol and cholesteryl esters (CEs). In normal cells and tissues *de novo* cholesterol biosynthesis is under tight feedback regulation [1]. It has been suggested that this mechanism might be lost during neoplasia (reviewed in [2]), probably a reflection of the high cholesterol requirements of actively dividing tumour cells.

Whereas high levels of high-density lipoprotein (HDL) are clearly associated with a decreased risk for developing coronary artery disease (due to HDL-mediated removal of peripheral cholesterol), it has been suggested that high levels of HDL are associated with an increased risk of breast cancer development [3,4]. It has been reported that the proliferation rates of immortalized human breast cancer cells are increased by the presence of HDL in the culture medium, an effect that is more pronounced for oestrogen-receptor-positive cells as compared with oestrogen-receptor-negative cell lines [5].

Cellular uptake of exogenous cholesterol is facilitated by lipoprotein receptors, e.g. the 'classical' low-density-lipoprotein (LDL) receptor and a scavenger receptor of the B class (SR-BI), which was recently identified as an HDL receptor in rodents [6,7]. The major mechanisms by which HDL-associated CEs are transferred from the lipoprotein to the target cell are fundamentally different from the receptor-mediated endocytosis pathway (holoparticle uptake) used for the delivery of LDL cholesterol to the cell. The former mechanism, termed selective uptake of HDL-CE, involves HDL binding to a specific receptor, transfer of lipids (but not proteins) to the cell, and subsequent dissociation of CE-depleted HDL particles from the cell [6]. Once HDL-CEs are internalized via the selective-uptake pathway, they are rapidly hydrolysed in an extralysosomal compartment [8,9], probably by hormone-sensitive lipase (HSL [10]). The major expression sites of SR-BI are the liver and steroidogenic tissues, in which its expression is regulated by trophic hormones [11–13]. The tissue-specific expression of SR-BI reflects its involvement in the removal of excess cholesterol from peripheral tissue to the

Abbreviations used: ACTH, adrenocorticotropic hormone; CE, cholesteryl ester; CLA-1, CD-36 and LIMPII analogous 1; HDL, high-density lipoprotein; HDL_3, HDL subclass 3; HSL, hormone-sensitive lipase; LDL, low-density lipoprotein; SR-BI, scavenger receptor class BI; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; LPDS, lipoprotein-depleted serum; $[^3H]Ch_{16:0}$, [^{3}H]cholesteryl palmitate; SREBP, sterol-regulatory-element-binding protein.

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liver (reverse cholesterol transport [14,15]) and as a receptor providing cholesterol in the form of CEs for hormone synthesis [16]. In more general terms, high SR-BI expression was found in a variety of cell models known to use large quantities of HDL-derived cholesterol [6,12,17]. Facilitation of bi-directional cholesterol flux is one of the unique features of SR-BI [18]. The human analogue of SR-BI, CLA-1 (CD36 and LIMPII analogous-1), has been identified as a member of the *CD36* gene family and is widely expressed [19]. Human CLA-1 and mouse SR-BI are highly similar (79 % amino acid sequence identity) [20] and share comparable biochemical functions and ligand-binding specificity [6,21,22].

To address more directly the role of exogenous HDL on proliferation rates of human breast carcinoma cells, we have investigated how HBL-100 cells acquire HDL-derived cholesterol. Our data indicate that CLA-1-mediated selective uptake of HDL-CEs and subsequent hydrolysis by HSL could provide a substantial contribution to the 'free' cholesterol pool in rapidly dividing HBL-100 breast carcinoma cells.

MATERIALS AND METHODS

Cell culture

During the present study HBL-100 human breast carcinoma cells were used and cultured as described in [23]. HBL-100 cells express the oestrogen and the LDL receptors [24]. Briefly, cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) fetal calf serum (FCS) or lipoprotein-depleted serum (LPDS), 2 mM L-glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin. Cells were cultured as monolayers in a humidified atmosphere of 95 % air/5 % CO_2 at 37 °C, and transferred or harvested at 70 % confluency by addition of 0.25 % trypsin, containing 1 mM EDTA, until the cells could be dislodged by tapping. Trypsin was inactivated by the addition of a 10-fold excess of fresh DMEM, and the trypsin/medium mixture was removed by low-speed centrifugation. Culture medium was changed routinely every 2 days.

Isolation of human plasma lipoproteins

Human HDL_3 (HDL subclass 3, $d = 1.125$–1.21 g/ml; containing apolipoprotein A-I as the major apolipoprotein) was prepared from fresh human plasma from normolipaemic donors by ultracentrifugation in a KBr gradient as described in [25]. After ultracentrifugation, the lipoproteins were dialysed against PBS (10 mM phosphate, pH 7.4, containing 150 mM NaCl) and stored at +4 °C under nitrogen. The protein content was determined according to Lowry et al. [26] using BSA as standard.

Labelling of lipoproteins

HDL_3 was iodinated with $Na^{125}I$ using *N*-bromosuccinimide as the oxidizing agent [27], resulting in specific radioactivities between 200 and 400 c.p.m./ng of protein. Labelling of the HDL_3-CE moiety was performed with [^{3}H]cholesteryl palmitate ([^{3}H]$Ch_{16:0}$) essentially as described in [28]. Tracer (200 μCi in toluene) and 140 μg of phosphatidylcholine (in trichloromethane) were dried under N_2 in a round-bottomed flask (25 ml) and 1 ml of PBS (pH 7.4) was added to the flask (flushed with argon), which was kept rotating at 37 °C to resuspend the lipids. After 30 min the flask was sonicated for 10 min under argon in a bath-type sonicator and transferred to a Pyrex tube containing 960 μl of LPDS and 6 mg of HDL_3. The mixture was incubated at 37 °C (overnight, under N_2). Labelled HDL_3 was isolated by ultracentrifugation as described above and excess KBr was removed by size-exclusion chromatography on PD10 columns (Pharmacia, Vienna, Austria). Specific radioactivities of [^{3}H]$Ch_{16:0}$-labelled HDL_3 were between 6 and 10 c.p.m./ng of protein.

Modification of lipoproteins

LDL and HDL_3 were oxidized in the presence of Cu^{2+} as described in [29]. Formation of conjugated dienes was assayed on-line by the increase in absorbance at 234 nm (within the first 3 h, $\Delta A_{234} \approx 0.8$–$0.9$). After 24 h, oxidation was stopped by the addition of EDTA (final concentration, 1 mg/ml). Acetylation of LDL was performed as described in [30]. Briefly, 1 ml of a solution containing 2.5 mg of LDL protein was added to 1 ml of a solution of saturated sodium acetate with continuous stirring on ice. Multiple 2-μl aliquots of acetic anhydride were added to the solution containing LDL in 2-μl aliquots within 1 h to give a mass 1.5 times higher than that of LDL. The solution was mixed for an additional 30 min, and dialysed against PBS.

Preparation of phosphatidylserine liposomes

Phosphatidylserine or phosphatidylcholine and cholesterol (all stock solutions 10 mg/ml chloroform) were mixed in a 1:1 molar ratio, the solvent was evaporated on a Rotavapor and the lipids were dried overnight. Tris/HCl (2 ml, 10 mM, pH 8.0, containing 0.1 M KCl and 1 mM EDTA) was added and kept rotating for 30 min at 37 °C. This mixture was sonicated for 5 min on ice, and dialysed against PBS. The phospholipid and cholesterol contents were analysed using commercially available enzymic test kits.

Determination of cholesterol in cellular lipid extracts

During all experiments the cells were counted in a haemocytometer in duplicate. After counting, the cellular lipids were extracted twice with 500 μl of hexane/2-propanol (3:2, v/v). After centrifugation at 14000 ***g***, 500 μl of the organic phase was removed and hydrolysed in KOH [31]. The neutral lipids were extracted into hexane, dried and converted into the corresponding trimethylsilyl ether derivatives [80 μl of acetone/20 μl bis(trimethylsilyl)trifluoroacetamide containing 1 % trimethylchlorosilane; 15 min at 50 °C]. Lipid extracts without prior hydrolysis were processed as described above to determine the intracellular unesterified cholesterol content. The difference between the cholesterol contents of hydrolysed versus non-hydrolysed samples should reflect the contribution of selective CE uptake to the total cellular cholesterol content. The trimethylsilyl-ether derivatives were analysed by GC and quantified with 5α-cholestane as an internal standard [32].

Intracellular hydrolysis of HDL_3-CEs

After incubation of HBL-100 cells in the presence of [^{3}H]$Ch_{16:0}$-labelled HDL_3 the cellular lipids were extracted as described above, dried under N_2 and resuspended in 150 μl of the mobile phase (hexane/ethanol/acetic acid; 98:1.995:0.005, by vol.). The cholesterol and CE contents in the samples were analysed on an EXSil100 silica column (5 μm of material, 200 mm × 4.6 mm; Activon, Sydney, Australia) at a flow rate of 1 ml/min on a Waters HPLC system (Waters, Vienna, Austria). Elution of [^{3}H]cholesterol and [^{3}H]$Ch_{16:0}$ was monitored by radiometric detection (RadioMatic Flow One/Beta; Packard-Canberra, Vienna, Austria) at a liquid-scintillator flow rate of 2 ml/min.

Cell experiments

Prior to the uptake experiments the cells were preincubated (overnight) in DMEM containing LPDS (10 %, v/v). Lipoprotein-uptake studies were performed in the presence of the

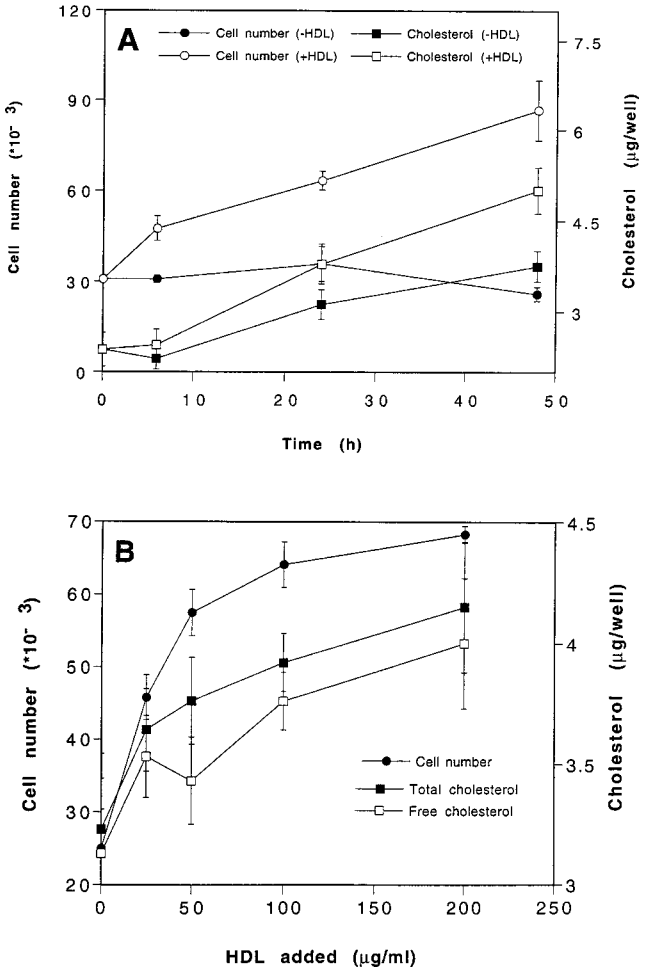

Figure 1 Effect of exogenous HDL_3 on HBL-100 growth rates and cellular cholesterol levels

(**A**) Cells (30000) were seeded in 6-well cluster trays and incubated in DMEM containing LPDS (10%; ■, ●) or in DMEM containing LPDS and 100 μg/ml HDL_3 (□, ○). At the indicated time points the cells were washed twice with PBS, trypsin-treated, and counted in a haemocytometer (○, ●) after 6, 24 and 48 h. The cellular cholesterol content was analysed by GC prior to (□) and after (■) hydrolysis of the cellular lipids in ethanolic KOH, as described in the Materials and methods section. Quantification was performed using 5α-cholestane as an internal standard. (**B**) Cells (30000) were seeded in 6-well cluster trays and incubated in DMEM containing LPDS (10%) in the presence of the indicated concentrations of HDL_3. After 48 h, cell numbers (●) and the cellular cholesterol content (□, ■) were determined. Data shown represent means ± S.D. for three independent experiments.

indicated amounts of iodinated (^{125}I-HDL_3) or [^{3}H]$Ch_{16:0}$-labelled HDL_3 for the indicated times, the medium was aspirated and the cells were washed twice with Tris/HCl (50 mM/150 mM NaCl, pH 7.4/2% BSA) and twice with the same buffer without BSA. Cells were then lysed in NaOH (0.3 M) and the radioactivity was counted. An aliquot of the hydrolysate was used to determine the cellular protein content [26]. In this study ^{125}I-HDL_3 uptake is termed holoparticle uptake, while [^{3}H]$Ch_{16:0}$-labelled HDL_3 uptake is referred to as apparent particle uptake (i.e. the amount of lipoprotein uptake that would account for [^{3}H]$Ch_{16:0}$ uptake is calculated from the specific radioactivity). This is necessary to be able to compare the two uptake pathways quantitatively. Selective uptake is calculated as the difference between apparent particle uptake and holoparticle uptake.

To determine intracellular hydrolysis rates of [^{3}H]$Ch_{16:0}$, the cellular lipids were extracted with hexane/2-propanol (2 × 1 ml), dried and analysed by HPLC with radiometric detection as described above. Competition experiments were performed in the presence of the indicated excess of the corresponding competitor. Binding constants were calculated by non-linear regression analysis using GraphPad.

Northern-blot analysis

RNA was isolated by the RNeasy Midi-kit (Qiagen, Vienna, Austria) from 2×10^6 HBL-100 cells. RNA (10 μg) was denatured at 65 °C for 5 min in sample loading buffer (20 mM Mops/ethidium bromide) and run through 1% agarose/20 mM Mops/8% formaldehyde gels (80 V) using 20 mM Mops as running buffer. The RNA was transferred to Hybond-N membrane (Amersham, Vienna, Austria) overnight in 10 × SSC (0.15 M NaCl/0.015 M sodium citrate), and fixed for 30 s under UV light. The membrane was probed with ^{32}P-labelled full-length rat SR-BI cDNA [33] overnight at +62 °C in 50% formamide, 5 × SSPE (50 mM sodium phosphate, pH 7.4/750 mM NaCl/5 mM EDTA), 0.01% BSA and 1% SDS, and washed in 0.5 × SSC/0.1% SDS at room temperature. The film was exposed for 5 days at −70 °C.

Western-blot analysis

Analysis of CLA-1 expression

HBL-100 cells were cultured in a T75 flask and incubated in DMEM containing FCS (10%, v/v) or LPDS (10%, v/v) overnight. The cells were washed twice with PBS (pH 7.4) containing 150 mM NaCl, 1 mM benzamidine, 20 μg/ml leupeptin and 20 μg/ml antipapain and scraped into 500 μl of the same buffer. Cells were sonicated on ice (10 s, 3 times) and centrifuged at 10000 ***g*** for 10 min. The supernatant (100 μl) was mixed with 100 μl of sample buffer, heated to 98 °C (3 min) and 20 μl of the mixture was applied to a SDS/PAGE gel (8%) and transferred electrophoretically on to nitrocellulose (150 mA, 4 °C, 90 min). Immunoreactive bands were detected with a polyclonal rabbit antiserum raised against a keyhole-limpet haemocyanin-coupled peptide corresponding to the cytoplasmic 15 C-terminal amino acids of SR-BI of the chinese hamster. Due to the sequence similarity in the C-terminal portion between SR-BI and CLA-1, the antibody also cross-reacts with CLA-1. Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as a secondary antibody and visualization of immunoreactive bands was performed using the ECL® detection system (Amersham).

Analysis of HSL expression

HBL-100 cells of one T75 flask were washed twice with ice-cold PBS, scraped, centrifuged at 4000 ***g*** (10 min) and the pellets frozen at −70 °C. For electrophoresis the cells were resuspended in 200 μl of homogenizing buffer (20 mM Tris/HCl/0.25 M sucrose/1 mM dithiothreitol/1 mM EDTA/1 mM benzamidine/20 μg/ml leupeptin/20 μg/ml antipapain, pH 7.4). Cells were lysed by disruption with sonication (10 s, 3 times) and the homogenate was centrifuged for 10 min at 10000 ***g***. For SDS/PAGE, 50 μl of SDS buffer and 12.5 μl of 10% SDS were added to 50 μl of supernatant, boiled for 5 min, separated on a 7.5% SDS gel and transferred on to nitrocellulose as described above. For immunoblotting polyclonal chicken anti-rat HSL (a kind gift of C. Holm, Department of Cell and Molecular Biology, Lund University, Lund, Sweden), rabbit anti-chicken IgG (Sigma) and alkaline phosphatase-conjugated affinity-purified goat anti-rabbit IgG (Sigma) were used. Homogenized mouse

epididymal fat pads containing relatively high concentrations of HSL were used as a positive control.

RESULTS

Effects of exogenous HDL_3 on HBL-100 growth rates

The time- and concentration-dependent effect of exogenously added HDL on HBL-100 cell growth is shown in Figure 1. HBL-100 cells (30000 cells) were seeded in 6-well trays and incubated in DMEM (containing 10% LPDS) in the absence or presence of human HDL_3 (100 μg/ml medium) for 6, 24 and 48 h. The addition of HDL_3 to the growth medium exhibited pronounced growth-stimulatory effects (an increase in cell numbers from 30000 to 85000 cells; Figure 1A). In contrast, cells cultured in LPDS-containing medium without HDL were in growth arrest. Also, the cellular cholesterol content was affected by the presence of HDL_3 in the culture medium. While the total cholesterol content of cells cultivated in the absence of HDL_3 increased from 2.3 to 3.8 μg of cholesterol/well (0 versus 48 h), the corresponding values for cells cultured in HDL_3-containing medium increased from 2.4 to 5.0 μg of cholesterol/well (Figure 1A). Proliferation rates of HBL-100 cells were dependent on the concentration of HDL_3 present in the culture medium (Figure 1B). Cell numbers increased with increasing HDL_3 content (from 30000 to 68000; 0–200 μg of HDL_3/ml). Also the cellular total cholesterol content increased from 3.2 to 4.2 μg of cholesterol/well in a manner dependent on the amount of exogenous HDL_3. When the cellular lipid extracts were analysed for the unesterified cholesterol content (i.e. without hydrolysis prior to derivatization and GC analysis) we have obtained consistently lower cholesterol concentrations (from 3.13 to 4.0 μg/well). These were, however, statistically not significantly different from values obtained from hydrolysed samples (Figure 1B, open versus filled squares). Whether this is a reflection of the relatively low capacity of the plasma-membrane bilayer for CE accommodation and rapid intracellular hydrolysis is presently not clear. However, from these experiments it is evident that the presence of HDL_3 in the culture medium affects both cell growth and the cholesterol content of HBL-100 cells.

Identification of CLA-1 and HSL by Northern- and Western-blot analyses

One mechanism that could contribute to HDL-mediated growth stimulation is the selective uptake of HDL-CE via CLA-1 and subsequent intracellular hydrolysis. This process would result in the generation of 'free' cholesterol in addition to the endogenously synthesized pool that could be utilized for the generation of new membrane surface area for rapidly dividing carcinoma cells.

In Northern blots the CLA-1 message was detected as a 2.8-kb species in both LPDS- and FCS-incubated cells with similar intensities (Figure 2A, lanes 1 and 2, respectively). In Western blots we could detect two bands in lysates of HBL-100 cells that were precultured in LPDS- (Figure 2B, lane 1) and FCS-containing medium (Figure 2B, lane 2). The apparent molecular masses of the immunoreactive bands were 82 and 78 kDa, respectively. The intensities of both bands were slightly higher (approx. 1.5-fold, as evaluated by densitometry) when cells were preincubated in LPDS in comparison with FCS. As a positive control for SR-BI expression we have used a rat liver homogenate (Figure 2B, lane 3).

One candidate that could facilitate intracellular hydrolysis of HDL-CEs which were taken up selectively is HSL. In line with intracellular CE hydrolysis within the 6-h time course (see below), we could detect a protein with an apparent molecular mass of 86 kDa using a polyclonal chicken anti-rat HSL as primary antibody (Figure 2C, lane 1). The apparent molecular mass of mouse fat HSL (Figure 2C, lane 2) was slightly lower (84 kDa), in line with data reported for HSL in different species [34].

Uptake mechanisms of HDL_3-CEs

In principle, cells can acquire HDL-associated lipids by either uptake of intact lipoprotein particles (holoparticle uptake) or a

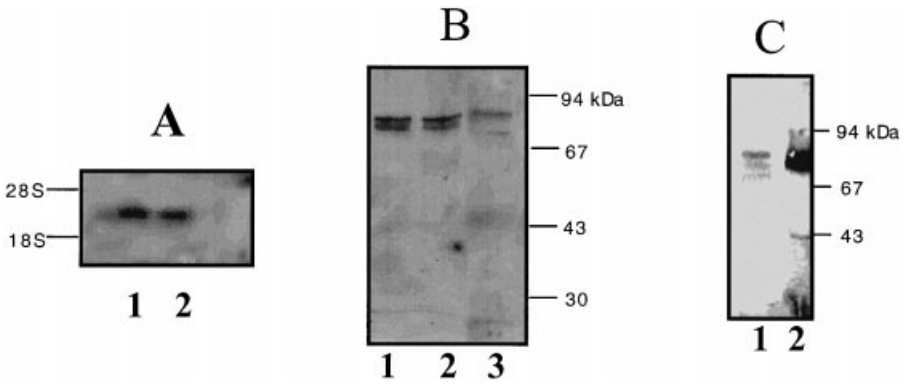

Figure 2 Northern- and Western-blot analyses of CLA-1 and HSL

(**A**) Northern-blot analysis of CLA-1 mRNA expression. RNA from HBL-100 cells cultured in DMEM containing LPDS (10%; lane 1) or FCS (10%; lane 2) was isolated, transferred to a Hybond membrane, and probed with a ^{32}P-labelled full-length rat SR-BI cDNA as described in the Materials and methods section. The migrations of 28 S and 18 S ribosomal RNAs are indicated. (**B**) Western-blot analysis of CLA-1 expression. HBL-100 proteins were separated on an SDS/PAGE gel (8%) under reducing conditions and transferred to nitrocellulose. CLA-1 was identified using a rabbit polyclonal antiserum (dilution, 1:1000) raised against a peptide corresponding to amino acids 496–508 of SR-BI. After addition of a peroxidase-conjugated goat anti-rabbit IgG (1:2000), immunoreactive bands were visualized with the ECL system. Prior to immunoblotting experiments, HBL-100 cells were cultured overnight in DMEM containing LPDS (10%; lane 1) or FCS (10%; lane 2). A rat liver homogenate was included as a positive SR-BI control (lane 3). Visualization was performed with the ECL method. (**C**) Western-blot analysis of HSL expression. HBL-100 cells were lysed as described in the Materials and methods section, separated on an SDS/PAGE gel (7.5%), and transferred to nitrocellulose. Western-blot analysis was performed with a polyclonal chicken anti-rat HSL primary antibody, followed by rabbit anti-chicken IgG and alkaline phosphatase-conjugated affinity-purified goat anti-rabbit IgG as secondary antibodies. Prior to Western blotting, HBL-100 cells were cultured overnight in DMEM containing FCS (lane 1). An infranatant of a homogenized mouse epididymal fat pad was used as positive HSL control (lane 2). For (**B**) and (**C**), the positions of the molecular-mass markers are indicated.

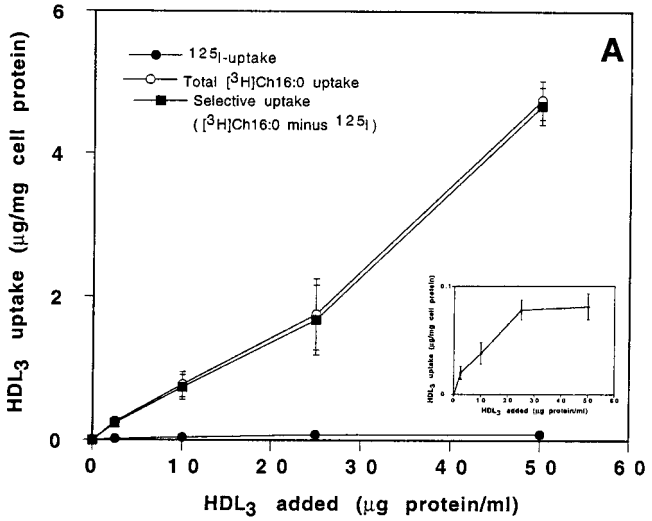

Figure 3 Uptake (A) and intracellular hydrolysis (B) of HDL$_3$-CE

(**A**) HBL-100 cells were seeded on 6-well plates and grown in DMEM/10% LPDS for 24 h before addition of HDL$_3$. The cells were then incubated for 6 h in the presence of increasing concentrations of ^{125}I-HDL$_3$ or [^{3}H]Ch$_{16:0}$-labelled HDL$_3$ in the absence or presence of a 10-fold excess of HDL$_3$ to differentiate between specific and non-specific uptake. Holoparticle, apparent particle and selective uptake was calculated as described in the Materials and methods section. After 6 h the medium was removed and the cells were washed, and analysed for radioactivity as described in the Materials and methods. HDL$_3$ uptake was calculated from the specific radioactivities (c.p.m./ng of protein) of the lipoprotein preparations and the cell-associated radioactivity. The inset shows uptake of ^{125}I-HDL$_3$ with the *y* axis drawn on a smaller scale (0–0.1 μg/mg of cell protein), and the *x* axis the same as the larger graph. Results shown are specific uptake data and represent mean values ± S.D. from three independent experiments. (**B**) HBL-100 cells were grown on 6-well plates for 24 h in DMEM containing LPDS and increasing amounts of [^{3}H]Ch$_{16:0}$-labelled HDL$_3$ were added to the medium. The cells were incubated for 6 h, washed, extracted and analysed for radioactive CE and cholesterol by HPLC with radiometric detection as described in the Materials and methods section. Data shown represent means ± S.D. for three independent experiments.

combination of holoparticle-dependent and holoparticle-independent (selective) uptake. To compare the relative contribution of holoparticle and selective HDL-CE uptake with total CE uptake, HBL-100 cells were incubated in the presence of increasing concentrations of ^{125}I-HDL$_3$ and [^{3}H]Ch$_{16:0}$-labelled HDL$_3$. Results of these experiments are shown in Figure 3(A).

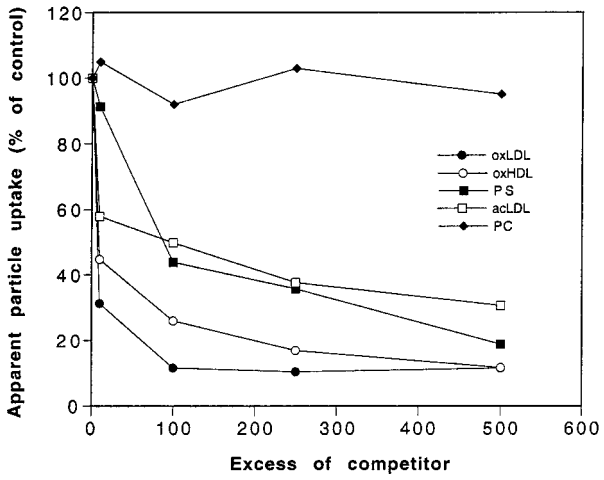

Figure 4 Competition of HDL$_3$ uptake by HBL-100 cells

HBL-100 cells were grown on 6-well plates for 24 h in DMEM containing LPDS before the competition experiments. To each well, 10 μg of [^{3}H]Ch$_{16:0}$-labelled HDL$_3$ together with the indicated molar excess of competitors was added. The excess of phosphatidylserine (PS) and phosphatidylcholine (PC) liposomes was based on the phospholipid content of HDL$_3$. After a 6-h incubation the cells were washed, lysed and the radioactivity was measured on a β-counter. Data shown represent means for three independent experiments (for more clarity error bars are not displayed; S.D. < 11%). oxHDL and oxLDL, oxidized HDL and LDL; acLDL, acetylated LDL.

HDL$_3$ holoparticle uptake provided only a small contribution to total HDL$_3$-CE uptake (between 2 and 8%), with a higher contribution at lower HDL$_3$ concentrations. Calculation of binding constants for ^{125}I-HDL$_3$ by non-linear regression analysis revealed a K_d of 28 μg of total HDL$_3$/ml (1.4×10^{-7} M) and a B_{max} of 220 ng of total HDL$_3$/mg of cell protein. The remaining HDL$_3$-CE uptake was attributable to selective uptake, exceeding HDL$_3$ holoparticle uptake by between 12- (2.5 μg/ml HDL$_3$) and 58-fold (50 μg/ml HDL$_3$). These findings underline the pronounced capacity of HBL-100 cells for selective HDL-CE uptake.

To investigate the intracellular fate of HDL$_3$-CEs taken up via the selective-uptake pathway, cells were incubated in the presence of [^{3}H]Ch$_{16:0}$-labelled HDL$_3$, and the cellular lipids were extracted and analysed by HPLC with radiometric detection as outlined in the Materials and methods section. As can be seen from Figure 3(B), HBL-100 cells acquired [^{3}H]Ch$_{16:0}$ in a dose-dependent manner in a near-linear process (1100–30000 c.p.m.). Part of the HDL$_3$-CE, which was taken up selectively, was subjected to intracellular hydrolysis, producing [^{3}H]cholesterol. Intracellular hydrolysis was dependent on the amount of HDL$_3$ present in the culture medium and decreased from 45 (2.5 μg) to 13% (50 μg) of total cell-associated [^{3}H]Ch$_{16:0}$. Thus, selective uptake of HDL$_3$-CEs results in intracellular accumulation of unesterified cholesterol within the 6-h incubation period studied here.

Uptake specificity of HDL$_3$-CEs by HBL-100 cells

To verify further that CLA-1 is responsible for selective HDL-CE uptake by HBL-100 cells, we have performed a series of competition experiments with native and modified lipoproteins that are known substrates for CLA-1 (Figure 4). The most efficient competitors for HDL$_3$-CE uptake were oxidized LDL and HDL (> 50% inhibition at a 10-fold excess of competitors), followed by acetylated LDL (50% competition at a 100-fold excess). As anionic phospholipids like phosphatidylserine are also ligands for CLA-1, we have included phospholipids in these

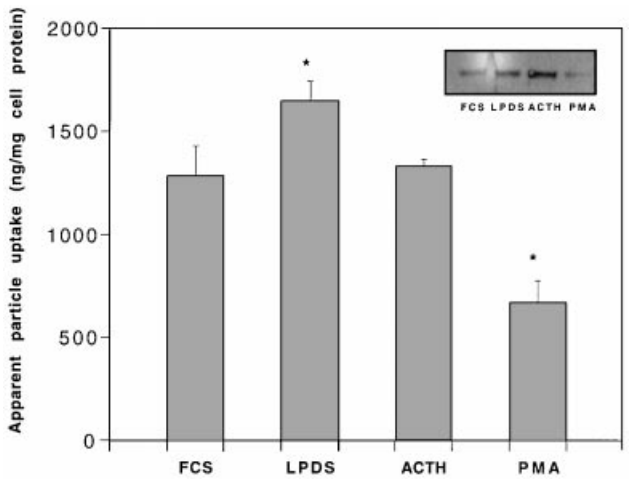

Figure 5 Regulation of HDL_3-CE uptake

HBL-100 cells were incubated overnight on 6-well plates in DMEM containing FCS (10%; v/v) or LPDS (10%; v/v) or DMEM/LPDS containing adrenocorticotropic hormone (ACTH; 1 μM) or PMA (100 nM). To each well, 20 μg of [^{3}H]$Ch_{16:0}$-labelled HDL_3 was added, the cells were incubated for 6 h, washed, lysed and counted in a β-counter. Data shown represent means $\pm$ S.D. for three independent experiments. $^*P < 0.001$, calculated by the Student's *t* test of independent samples; FCS versus LPDS, ACTH, PMA. The insert shows Western-blot analysis of CLA-1 protein expression after the corresponding treatments (with FCS, LPDS, ACTH and PMA).

competition studies. Phosphatidylserine competed for approx. 80% of selective HDL_3-CE uptake, whereas the addition of phosphatidylcholine did not impair selective HDL_3-CE uptake. This latter result is also indicative for CLA-1-mediated selective uptake of HDL_3-CE by HBL-100 cells.

It has been suggested that cellular levels of free cholesterol regulate SR-BI expression via a feedback loop [35]. To test whether HDL_3-CE uptake by HBL-100 cells is regulated by the cholesterol content of the medium, cells were preincubated in DMEM containing either FCS or LPDS (Figure 5). Incubation in LPDS-containing medium significantly up-regulated HDL_3-CE uptake (1280 versus 1650 ng/mg of cell protein; FCS versus LPDS conditions), findings in line with sterol-regulatory-element-binding protein (SREBP)-1a-regulated transcription of the *SR-BI* gene [36]. We have also investigated the effects of adrenocorticotropic hormone (ACTH) and PMA (Figure 5) on the efficacy of HDL_3-CE uptake by HBL-100 cells, since both agents are known to regulate SR-BI and CLA-1 expression [21,35]. While ACTH was without effect, PMA treatment of the cells significantly reduced the capacity for HDL_3-CE uptake (1330 and 668 ng/mg of cell protein; ACTH and PMA, respectively). The inset of Figure 5 shows Western-blot analysis of membrane-protein fractions obtained from the correspondingly treated cells. Densitometric evaluation of immunoreactive bands revealed that CLA-1 was up-regulated by LPDS and down-regulated by PMA (inset to Figure 5, lanes 2 and 4, respectively), reflecting the findings for HDL-CE uptake. In contrast, ACTH treatment up-regulated CLA-1 expression (Figure 5 inset, lane 3); however, this was not accompanied by increased uptake of HDL_3-CE.

DISCUSSION

In the present study we have investigated whether human HBL-100 cells acquire cholesterol from exogenous human HDL via the selective-uptake pathway. From our results it is conceivable that CLA-1-mediated selective uptake of HDL-CE and subsequent hydrolysis by HSL could contribute significantly to an accessible 'free' cholesterol pool. This pool might be used for the generation of new membrane surface area by rapidly dividing tumour cells or facilitate intracellular signalling pathways involved in the regulation of cell proliferation [37].

A number of studies have reported that cancer cells and solid tumours take up LDL more effectively than normal tissues (reviewed in [38]), which is probably a reflection of a higher cholesterol demand of dividing cells in contrast with differentiated cells. On the other hand, some tumours do not internalize great amounts of LDL. Thus it has been suggested that is important to show not only binding but also internalization of LDL [38]. While this is certainly true for LDL-based drug-targeting studies, the demonstration of internalization might be insufficient regarding cholesterol utilization by tumour cells. As a result of selective LDL-CE uptake (e.g. [39,40]), the utilization of LDL-derived cholesterol/CEs might be underestimated by the analysis of LDL holoparticle internalization.

It is interesting that HDL-derived cholesterol has received much less attention. This might be due to the fact that an HDL receptor was identified only recently [6]. In these studies, it was demonstrated that SR-BI mediates selective uptake of HDL-CE, is expressed in a tissue-specific manner and is under tight hormonal regulation [16]. The human homologue of SR-BI, CLA-1, shares $\approx 80\%$ amino acid similarity with SR-BI, mediates comparable functions as does the rodent receptor [21,22], and is expressed predominantly in human tissues performing very active cholesterol turnover, i.e. the liver and steroidogenic tissues [22]. During the present study with HBL-100 cancer cells we have observed exceptionally high selective uptake of HDL-CEs. In our experiments selective HDL-CE uptake exceeded HDL holoparticle uptake by between 12- and 58-fold. In contrast with ^{125}I-HDL_3 holoparticle binding, selective HDL_3-CE uptake was not a saturable process, a phenomenon consistently observed during different studies (e.g. [6,41,42]). This might be a reflection of a continuous CE influx into the cells fuelled by dissociation of CE-depleted HDL particles from, and rebinding of HDL particles with 'normal' CE content to, CLA-1.

Northern- and Western-blot analyses have verified that HBL-100 cells express CLA-1, the human receptor facilitating selective HDL-CE uptake (see above). Although we could detect only a single 2.8-kb RNA species in Northern blots, Western-blotting experiments revealed other immunoreactive bands with molecular masses of 82 and 78 kDa. It is presently not clear whether or not this is a reflection of alternative mRNA splicing of a single precursor transcript, as reported for SR-BI/SR-BII biosynthesis [41,43]. The specificity of selective HDL-CE uptake by HBL-100 cells is similar to the ligand-binding specificity described for CLA-1 [21,22]. Selective uptake of HDL-CEs was up-regulated by depletion of intracellular cholesterol pools and down-regulated by PMA treatment, findings in line with SR-BI expression reported for hepatocytes [13,42] and CLA-1 expression by human monocytic THP-1 cells [21], respectively.

According to our findings, selective uptake of HDL-CEs via a CLA-1-mediated pathway could indicate the first step in providing cholesterol to HBL-100 cells, probably in a similar manner to that suggested for developing embryonic tissues [44]. However, it is clear that CEs must be hydrolysed by an intracellular esterase to be available for incorporation into newly formed membranes. CEs that have been taken up via the selective-uptake pathway are hydrolysed in an extralysosomal compartment, probably the cytosol [8,9]. One potential candidate that could

facilitate CE hydrolysis after selective uptake is the neutral, multifunctional esterase HSL [45]. In addition to triacylglycerol lipase activity, HSL mediates neutral CE hydrolase activity [10,45]. Co-expression of high levels of SR-BI [6] and HSL [46] in steroidogenic tissues could reflect a functional relationship between these proteins in tissues with high cholesterol-turnover rates. The expression of HSL by HBL-100 cells could provide a plausible explanation for the relatively high intracellular hydrolysis rates of internalized CEs. Whether this is a reflection of a comparable inter-relationship between selective CE uptake via CLA-1 and subsequent hydrolysis by HSL in cancer cells remains to be established. It is important to note that a number of oestrogen-receptor-positive and oestrogen-receptor-negative human breast cancer cell lines express the peripheral-type benzodiazepine receptor (PBR) [37]. PBR facilitates transport of unesterified cholesterol into the nucleus, an observation associated with increased cellular proliferation rates of MDA-231 breast cancer cells [37]. As outlined in [37], it is not clear whether PBR-mediated cholesterol transport into the nucleus involves signalling via SREBPs.

An important question arising from our study is whether or not CLA-1 expression could be used for lipoprotein-mediated drug targeting in a similar manner as reported for the LDL receptor (reviewed in [38]). Lipoproteins and apolipoprotein-containing liposomes are applied as potential delivery systems for anti-tumour agents [47,48]. Lactosylated reconstituted HDL particles have been reported to be potential drug-carrier vehicles, which are subjected to hepatic uptake via the asialoglycoprotein receptor [48]. Incorporation of an elliptinium derivative into LDL improved the potency of this drug against B16 melanomas in mice [49]. As CLA-1 is a multi-ligand receptor, it could be useful to study HDL-mediated delivery of hydrophobic cytotoxic drugs which have a structural relationship to CEs.

There is general agreement that high HDL concentrations are associated with a decreased risk of the development of coronary artery disease [50], a process mediated in part by SR-BI [42,51–54]. However, the situation is less clear with regard to circulating HDL levels and the risk of developing breast cancer, a leading cause of death of women in Western societies. Some authors have reported an association between high HDL cholesterol and the incidence of breast cancer [3,4], a fact that is possibly related to oestrogen metabolism [55]. This could be a reflection of cholesterol supply via selective HDL-CE uptake (serving as precursors for hormone synthesis) or the result of other signalling pathways mediated by HDL [56,57].

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