Endothelial lipase-modified high-density lipoprotein exhibits diminished ability to mediate SR-BI (scavenger receptor B type I)-dependent free-cholesterol efflux

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Endothelial lipase (EL) is a phospholipase with little triacylglycerol lipase activity. To assess structural and functional properties of EL-HDL (EL-modified high-density lipoprotein), HDL was incubated with conditioned medium from Cos-7 cells infected with adenovirus encoding human EL. After re-isolation of HDL by ultracentrifugation, TLC and HPLC analyses revealed that EL-HDL was markedly depleted in phosphatidylcholine and enriched in lyso-phosphatidylcholine compared with LacZ-HDL (control HDL) incubated with conditioned medium from Cos-7 cells infected with adenovirus encoding β -galactosidase. The EL-HDL was enriched in non-esterified fatty acids and, as revealed by lipid electrophoresis, was more negatively charged than control HDL. The HDL particle size as well as the total cholesterol, free cholesterol and triacylglycerol content of HDL were not significantly altered after EL modification. The ability of EL-HDL to mediate ³H-cholesterol efflux from SR-BI (scavenger receptor B

type I) overexpressing Chinese-hamster ovary cells was impaired and markedly lower compared with LacZ-HDL at HDL concentrations of 100 μ g/ml and above. Studies with ¹²⁵I-labelled HDL showed almost unaltered binding affinity (K_m values) and a slightly but significantly decreased binding capacity (B_{max} values) of EL-HDL to SR-BI, compared with LacZ-HDL. The ATP-binding-cassette transporter A1-dependent cholesterol and phospholipid effluxes were not affected by EL modification. From these results, we concluded that EL modification alters chemical composition and physical properties of HDL, resulting in its decreased binding capacity to SR-BI and a diminished ability to mediate SR-BI-dependent cholesterol efflux.

Key words: adenoviral vector, cholesterol efflux, phospholipase, reverse cholesterol transport, scavenger receptor B type I (SR-BI), structure–function.

INTRODUCTION

Epidemiological studies have demonstrated that HDL (high-density lipoprotein) plasma levels are inversely correlated with the risk for coronary artery disease [1–3]. The anti-atherogenic property of HDL is mediated by its ability to remove an excess of FC (free cholesterol) from peripheral cells and deliver it to the liver in the process of reverse cholesterol transport [4]. The FC efflux from the cell membranes represents the first step of the reverse cholesterol transport pathway and was found to be augmented in cells expressing the HDL receptor SR-BI (scavenger receptor B type I) [5,6]. The efflux studies with the PC (phosphatidylcholine)-depleted or -enriched HDL, respectively, have shown that SR-BI-mediated FC efflux is a function of the PC content of HDL [7]. Additionally, studies with PC-depleted or -enriched HDL showed that the PC content of HDL markedly affects apoA-I conformation [8] and consequently the binding efficiency of HDL to SR-BI [9]. The efficient interaction between HDL and SR-BI was found to be a prerequisite for SR-BI-mediated FC efflux to HDL [10].

EL (endothelial lipase) is a member of the triacylglycerol lipase gene family synthesized by various tissues and cell types, including endothelial cells [11,12]. By its preferential phospholipase activity, EL very efficiently cleaves HDL-PL (where PL stands for phospholipid) [13–15] liberating fatty acids, which are taken up by EL-expressing cells and are incorporated into endogenously synthesized lipids [15]. We have recently shown that, by its bridging

function, EL facilitates HDL particle binding and uptake as well as selective uptake of HDL-CE (where CE stands for cholesteryl ester) in HepG2 cells infected with EL-expressing adenovirus [14]. Experiments in mice with the targeted native EL locus [16,17] as well as in transgenic mice harbouring the human EL gene (LIPG locus) [16] revealed an inverse relationship between HDL cholesterol level and EL expression.

The aim of the present study was to assess the structural and functional properties of *in vitro* EL-HDL (EL-modified HDL). We found that EL-HDL was depleted in PC, enriched in lyso-PC and NEFA (non-esterified fatty acid) and was consequently more negatively charged compared with control HDL. Cell-culture experiments revealed a decreased ability of EL-HDL to promote SR-BI-mediated ³H-cholesterol efflux as well as a slightly but significantly decreased binding capacity of ¹²⁵I-EL-HDL to SR-BI-overexpressing cells compared with control HDL.

EXPERIMENTAL

Isolation of human HDL

HDL (subclass 3, $d = 1.125$ – 1.21 g/ml) was prepared by sequential density ultracentrifugation of plasma obtained from normolipidaemic blood donors [18]. After ultracentrifugation, HDL was dialysed against PBS (pH 7.4) and the protein concentration was determined by the Lowry method [19].

Abbreviations used: ABCA1, ATP-binding-cassette transporter A1; apoA-I, apolipoprotein A-I; CE, cholesteryl ester; DIDS, 4,4'-di-isothiocyanostilbene-2,2'-disulphonate; DMEM, Dulbecco's modified Eagle's medium; EL, endothelial lipase; EL-Ad, (human) EL-expressing adenovirus; HDL, high-density lipoprotein; EL-HDL, EL-modified HDL; FC, free cholesterol; FCS, foetal calf serum; LacZ-HDL, control HDL; LDL, low-density lipoprotein; NEFA, non-esterified fatty acid; PC, phosphatidylcholine; PL, phospholipid; PLA₂, phospholipase A₂; PLA₂-HDL, PLA₂-modified HDL; RA, 9-*cis*-retinoic acid; SR-BI, scavenger receptor B type I.

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Cell-culture, recombinant adenovirus infection and EL expression

Cos-7 cells (6×10^5) were plated on to 60 mm dishes and incubated under standard conditions (37 °C, 5 % CO₂ and 95 % humidity) in DMEM (Dulbecco's modified Eagle's medium) containing 10 % (v/v) FCS (foetal calf serum). After 24 h, cells were infected with a MOI (multiplicity of infection) 200 of EL-Ad [(human) EL-expressing adenovirus] or control virus encoding β -galactosidase (EL-LacZ) as described previously [14,15]. After infection in the culture medium containing 2 % FCS for 120 min at 37 °C, cells were incubated for 3 h with a medium containing 10 % FCS. Thereafter, cells were extensively washed with PBS and incubated with 3 ml/dish of a serum-free medium Panserin 401 (Pan Biotech, Aidenbach, Germany) for 24 h under standard cell-culture conditions. Conditioned media obtained from EL-Ad and β -galactosidase-expressing adenovirus infected cells (EL- and LacZ-conditioned medium respectively) were collected in prechilled tubes and spun for 10 min in a bench centrifuge at 4 °C to remove the cell debris. Aliquots were stored at -70 °C until use for HDL modification.

EL modification of HDL

To assess the efficiency of HDL hydrolysis by EL, HDL (1 mg of protein/80 μ l) was mixed with EL-conditioned medium (320 μ l), and NEFA-free BSA (50 μ l, Sigma), which was prepared as a stock solution (100 mg/ml) in Panserin 401 medium and incubated in a water bath at 37 °C. As a control, HDL was incubated under identical conditions except that the LacZ-conditioned medium was used instead of the EL-conditioned medium. Aliquots (10 μ l) were removed from the incubation mixtures at the indicated time points and the activity of EL was monitored by measuring the release of NEFA using a commercial kit (NEFA-C; Wako, Neuss, Germany). To prepare EL- and LacZ-HDL (control HDL) for further analysis and cell-culture experiments, HDL (5.8 mg of protein/400 μ l) was mixed with 1.6 ml of EL- or LacZ-conditioned medium, respectively, and 220 μ l of NEFA-free BSA stock solution and incubated at 37 °C. The final concentration of NEFA-free BSA in all incubations was 1 %. After a 16 h incubation, each incubation mixture was diluted by PBS to a final volume of 5.2 ml and the density was adjusted to 1.21 g/ml by the addition of solid NaBr. EL- and LacZ-HDL were re-isolated as a top fraction after ultracentrifugation at 15 °C for 24 h at 232 243 g (rotor VTi 65.2, rotor diameter 180 mm; Beckman). After extensive dialysis against PBS (pH 7.4), EL- and LacZ-HDL were used for subsequent experiments.

Characterization of EL-HDL

Total cholesterol, unesterified cholesterol (FC), NEFA and triacylglycerols in EL- and LacZ-HDL were measured by commercially available kits (Chol from Roche Diagnostics, Mannheim, Germany; Free Cholesterol C and NEFAC from Wako; Triglycerides Enzymatique PAP 150 from bioMérieux, Marcy l'Etoile, France).

TLC of PLs

A total of 400 μ g of HDL protein was extracted using a method described by Bligh and Dyer [20]. The chloroform phase was dried in Speed Vac and the lipid phase was redissolved with 50 μ l of a chloroform/methanol (2:1) solution and applied on to a TLC plate. The lipids were separated using a mixture of chloroform, methanol, acetic acid and water (25:15:4:2, by vol.) as a mobile phase and visualized by spraying 10 % H₂SO₄ on to the plate

followed by heating at 120 °C for 15 min. A mixture of purified lyso-PC and PC (Sigma-Aldrich) was used as a standard.

HPLC of PLs

PL analysis of 500 μ g of EL- and LacZ-HDL proteins was performed on a Hewlett-Packard 1050 system, equipped with a Vorex light-scattering detector ELSD IIA with a Vertex column (LiChrospher Diol 100, 5 μ m, 250 $\times$ 2.0 mm; Knauer, Berlin, Germany). The atomizer inlet air flow (60 mm) and drift tube temperature (120 °C) of the detector were maintained throughout all analyses. Separations were performed by gradient elution (flow rate, 0.8 ml/min) using solvent systems of (A) n-hexane, (B) methanol/acetic acid/triethylamine (765.7:15.31:12.9, by wt.) and (C) acetone/acetic acid/triethylamine (765.4:15.31:12.9, by wt.) as described previously [21]. Eluents were flushed continuously with helium. PLs, including PC, phosphatidylinositol, phosphatidic acid, phosphatidylserine and phosphatidylethanolamine used for calibration curves were extracted from wild-type yeast cells grown in full medium. These lipids were separated by two-dimensional TLC on silica gel 60 plates by using chloroform, methanol and 25 % aqueous ammonia (13:7:1, by vol.) as solvents for the first dimension, and chloroform, methanol, acetic acid and water (10:4:2:2:1, by vol.) for the second dimension. Determination of PL content was performed by a standard phosphorus quantification procedure [22].

Agarose-gel electrophoresis

A 0.5 % agarose gel was prepared with 50 mM 5,5-diethylbarbituric acid sodium salt (pH 8.2). EL- and LacZ-HDL proteins (8 μ l each) were loaded on to the gel, and electrophoresis was performed in the running buffer (10 mM 5,5-diethylbarbituric acid/50 mM 5,5-diethylbarbituric acid sodium salt, pH 8.2) at 20 mA for 1 h under buffer cooling. The gel was fixed in 0.5 % sulphosalicylic acid for 15 min, and after air-drying overnight, the lipids were stained with 1 % sudan black in 60 % ethanol (absolute ethanol/water, 60:40, v/v) for 20 min. Proteins were stained with Thiazin Red in 1 % acetic acid for 5 min.

Dynamic light scattering

Dynamic light-scattering experiments were performed with EL- and LacZ-HDL. The laboratory-built goniometer was equipped with a diode-pumped laser (Verdi V-5 from Coherent Inc., Santa Clara, CA, U.S.A.; $P_{\max} = 5$ W, $\lambda = 532$ nm), single-mode fibre detection with a photomultiplier and an ALV-5000 correlator (ALV, Langen, Germany). Measurements were performed at a scattering angle of 90° at 25 °C and a laser power of 200 or 250 mW. The time dependence of the scattering intensity, represented by its correlation function, provides information on diffusional motion of the scatterer. The diffusion coefficient is related to the apparent hydrodynamic radius R_H by the Stokes-Einstein equation, i.e. an equivalent sphere with radius R_H shows the same diffusion behaviour as the particle under investigation and serves as a size parameter. Any increase or decrease in the size of HDL particles would lead to an increase or decrease in the measured R_H values.

Non-denaturing gradient-gel electrophoresis

Electrophoresis was performed using PhastSystem with precast 8–25 % non-denaturing polyacrylamide gels (PhastSystem and PhastGel; Amersham Biosciences, Uppsala, Sweden) at 8 °C. After a prerun (40 min at 125 V), 12 μ g of EL- and LacZ-HDL were loaded on to the gel and run for 30 min at 50 V, followed by

a 30 min run at 70 V and finally for 24 h at 150 V. The gels were fixed in sulphosalicylic acid for 5 min and stained with 0.04 % Coomassie R 350 (PhastGel Blue R; Amersham Biosciences) for 20 min. Destaining was performed in a mixture containing 20 % acetic acid/20 % methanol (v/v). The particle size of HDL was determined by comparison with standard proteins (HMW Native Marker kit; Amersham Biosciences).

Western-blot analysis

EL-HDL (45 µg of protein) was analysed by SDS/PAGE (12 % gel) and with subsequent immunoblotting using EL-specific antibody exactly as described in [14,15].

HDL labelling with Na¹²⁵I

Iodination of EL- and LacZ-HDL as performed as described previously [14] using *N*-Br-succinimide as the coupling reagent [23]. Briefly, 0.5 mCi of Na¹²⁵I (New England Nuclear, Stevenage, Herts., U.K.) was used to label 3 mg of HDL protein. Labelled HDL was stored at 4 °C under argon atmosphere and used within 1 week.

Cell-culture studies

The IdIA-7 cells and stably transfected IdIA-7 cells overexpressing murine SR-BI (IdIA[SR-BI]) were described previously [5,10]. The IdIA-7 cells were cultured in Ham's F12 medium containing 5 % FCS under standard cell-culture conditions, whereas the cultured medium of IdIA[SR-BI] cells contained additionally 0.5 mg/ml G418. Both cell lines were kindly provided by Dr Monty Krieger (MIT, Cambridge, MA, U.S.A.). Human monocyte-derived macrophage cell line, THP-1 cells were cultured in RPMI 1640 supplemented with 10 % FCS. To obtain differentiated macrophages, cells were cultured in the presence of 160 nM PMA for 72 h.

¹²⁵I-HDL association with IdIA-7 and IdIA[SR-BI] cells

Association studies of EL- and LacZ-HDL to IdIA-7 and IdIA[SR-BI] cells were performed in 24-well dishes in triplicate. Cells were washed with PBS and increasing amounts of labelled lipoproteins were then added to the DMEM without FCS (0.25 ml) and the cells were incubated for 2 h at 37 °C. After incubation, the cells were washed twice with Tris-buffered saline containing 0.2 % BSA followed by two washes with Tris-buffered saline. The cells were then lysed with 0.5 ml of 0.25 M NaOH at room temperature (20 °C). The cell-associated radioactivity and protein content [19] were measured in an aliquot of the cell lysate. SR-BI-specific association of EL- and LacZ-HDL was determined by subtracting values for IdIA-7 cells from IdIA[SR-BI] cells to generate an SR-BI association curve. Binding parameters for $B_{\max}$ and K_d values were obtained from the SR-BI curve by non-linear regression (Prism).

Cholesterol efflux

The IdIA-7 and IdIA[SR-BI] cells were labelled with 1 µCi/ml [³H]cholesterol (New England Nuclear) in the corresponding culture medium at 37 °C for 30 h. After labelling cells were washed twice with Tris-buffered saline containing 0.2 % (w/v) BSA and twice with Tris-buffered saline. Cells were then incubated with the indicated amounts of EL- or LacZ-HDL protein in DMEM without FCS or with DMEM alone. After 2 h, or at the indicated time points, the medium was collected and measured in a β-counter. Cells were lysed with 0.25 M NaOH to determine the cellular protein content and the cell-associated radioactivity. Total

cellular ³H-cholesterol was calculated as the sum of the radioactivity in the efflux medium plus the radioactivity in the cells and was used to calculate the ³H-cholesterol efflux (percentage of total ³H-cholesterol released into the medium). THP-1 cells (250 000/well) were plated on to 24-well dishes and incubated for 72 h in RPMI 1640 + 10 % FCS + 160 nM PMA. During the last 24 h, cells were labelled with 1 µCi/ml ³H-cholesterol and to stimulate the ABCA1 (ATP-binding-cassette transporter A1) expression, 10 µM RA (9-*cis*-retinoic acid) was added to the medium during the last 16 h of incubation. After 24 h labelling, cells were washed with PBS + 0.2 % BSA and further equilibrated in RPMI 1640 + 0.2 % BSA, containing 160 nM PMA and 10 µM RA for 2 h. Efflux was performed in the equilibration medium (250 µl/well) either in the absence or presence of the ABC transporter inhibitor, 0.8 mM DIDS (4,4'-di-isothiocyanostilbene-2,2'-disulphonate) and the indicated concentrations of EL- and LacZ-HDL proteins or EL- and LacZ-incubation mixtures containing defined amounts of HDL protein. At a concentration of 0.8 mM, DIDS decreased apoA-I (15 µg/ml)-mediated cholesterol efflux by 55 % (results not shown). Control experiments were also conducted in the absence of RA, yielding cells with a low-level ABCA1 expression as revealed by Western-blot analysis (results not shown) and a concomitantly low level of cholesterol efflux when apoA-I (15 µg/ml) was used as the acceptor (results not shown). After 3 h, the medium and cells were processed as described above for cholesterol efflux in Chinese-hamster ovary cells.

PL efflux

THP-1 cells were plated as described above for cholesterol efflux and labelled with ³H-choline (1 mCi/ml) in RPMI 1640 + 10 % FCS for 24 h. After labelling and 2 h equilibration in RPMI 1640 + 0.2 % BSA, efflux was calculated as described above for cholesterol efflux using EL- and LacZ-incubation mixtures as acceptors. After 3 h, the medium was collected, spun and extracted by the method of Bligh and Dyer [20]. Aliquots of the organic phase were washed with a methanol/water mixture and used for scintillation counting. Cellular lipids were extracted with hexane/propan-2-ol (3:2, v/v), dried and subsequently mixed with PBS and finally extracted as cell media by the method of Bligh and Dyer [20]. Aliquots were measured as described above for cholesterol efflux.

Statistics

Results are expressed as means ± S.D. Significance of differences was examined using Student's *t* test.

RESULTS

To study the properties of EL-HDL, HDL was incubated with EL-conditioned medium obtained from Cos-7 cells infected with the adenovirus encoding human EL (EL-Ad) or with control, LacZ-conditioned medium obtained from cells infected with the adenovirus encoding β-galactosidase. Incubation of HDL with EL medium resulted in the cleavage of HDL-PLs by EL phospholipase activity. Consequently, concentration of NEFA in the incubation mixtures increased as a function of the incubation time (Figure 1). The amounts of NEFA in the control incubations (HDL + LacZ medium) were constant and 5-fold lower compared with EL incubations after a 24 h incubation. To assess the impact of EL modification on charge alteration of HDL, we analysed the electrophoretic mobility of EL- and LacZ-HDL by agarose-gel electrophoresis. As shown in Figure 2, the mobility

Figure 1 Hydrolysis of HDL-PL by EL

HDL₃ was incubated with EL- (▲) or LacZ-conditioned (■) medium obtained from Cos-7 cells infected with adenoviruses encoding either human EL or β -galactosidase (control) in a water bath at 37 °C. Aliquots (10 μ l) were removed from the 450 μ l incubation mixtures containing 1 mg of HDL₃ and 1% NEFA-free BSA at the indicated time points. Activity of EL was monitored by measuring the release of NEFA using a commercial kit (NEFA-C; Wako). The results represent means $\pm$ S.D. for three independent experiments using different HDL preparations. *** $P \leq 0.001$ (compared with LacZ-conditioned medium); ** $P \leq 0.01$ (compared with LacZ-conditioned medium).

Figure 2 Agarose-gel electrophoresis of EL- and LacZ-HDL

EL- and LacZ-HDL proteins (8 μ g each) were loaded on to a 0.5% agarose gel prepared in 50 mM barbital buffer (pH 8.2). After electrophoresis, the gel was fixed and lipids and proteins were stained as described in the Experimental section.

of EL-HDL was increased compared with LacZ-HDL, suggesting an enrichment in negatively charged species in EL-HDL. Quantification of NEFA revealed an increased amount of NEFA in EL-HDL compared with LacZ-HDL (Figure 3A), providing an explanation for the observed difference in electrophoretic mobility between EL- and LacZ-HDL. To assess the impact of EL on the HDL-PL content, we analysed lipid extracts from EL- and LacZ-HDL by HPLC. After incubation of HDL with EL for 16 h at 37 °C, approx. 40% of the PC, which is the major PL species in HDL was hydrolysed (Figure 3A). TLC analysis of HDL-PL revealed that a decrease in the PC content was accompanied by an increase in the lyso-PC content. As shown in Figure 3(B), EL-HDL contained substantial amounts of lyso-PC, a species that could not be detected in LacZ-HDL. The amounts of triacylglycerols, total cholesterol and FC were not significantly altered after EL modification of HDL (results not shown). To determine whether depletion of PC by EL altered the size of HDL, EL- and LacZ-HDL were analysed by dynamic light-scattering and non-denaturing gradient-gel electrophoresis. As shown in Figure 4, the R_H values and the electrophoretic mobility of HDL in

Figure 3 Determination of NEFA, PC and lyso-PC content in EL- and LacZ-HDL

HDL₃ (5.8 mg of protein/400 μ l) was incubated with 1.6 ml of EL- (grey bars) or LacZ-conditioned (black bars) medium in the presence of 1% NEFA-free BSA at 37 °C for 16 h. After re-isolation by density gradient ultracentrifugation (A), the NEFA-content was determined by a commercially available kit from Wako and the PC-content was determined by HPLC of HDL lipids as described in the Experimental section. Results are the means $\pm$ S.D. for three (NEFA) and two (PC) independent experiments performed in triplicates using different HDL preparations and conditioned media. *** $P \leq 0.001$ (compared with LacZ-HDL); * $P \leq 0.05$ (compared with LacZ-HDL). (B) The lyso-PC content was analysed by TLC using aliquots of HDL lipids used for HPLC as described in (A). The lipids were separated using as mobile phase a mixture of chloroform, methanol, acetic acid and water (25:15:4:2) and visualized by 10% H_2SO_4 (concentrated H_2SO_4 /water solution, 10:90, v/v). This was obtained by dilution of H_2SO_4 (1.84 g/ml) with water (10:90, v/v) at 120 °C for 15 min. A mixture of purified lyso-PC and PC was used as a standard. Representative results from one of two independent experiments performed in triplicate are shown.

non-denaturing gradient-gel electrophoresis were not altered on EL modification. Considering the altered PL composition of EL-HDL, together with the fact that the HDL-PL composition is a major determinant of the FC flux from cells mediated by SR-BI, we attempted to assess the ability of EL-HDL to mediate 3H -cholesterol efflux from cells. For this purpose, IdIA-7 and IdIA[SR-BI] cells respectively, labelled with 3H -cholesterol, were incubated with EL- and LacZ-HDL for 2 h. We observed essentially no efflux from either IdIA-7 or IdIA[SR-BI] cells in the absence of lipoproteins as acceptors (results not shown). We also observed, in line with previous studies [10,24], that the rate of HDL-mediated cholesterol efflux from SR-BI overexpressing IdIA[SR-BI] cells was significantly greater than that from IdIA-7 cells (results not shown). Results presented in Figure 5 show that the ability of EL-HDL to mediate cholesterol efflux via SR-BI was impaired at all time points, but significantly only at concentrations of 100 μ g/ml and higher compared with LacZ-HDL. To examine

Figure 4 Determination of EL- and LacZ-HDL size by dynamic light-scattering (A) and non-denaturing gradient-gel electrophoresis (B)

EL- and LacZ-HDL were generated as described in the Experimental section. (A) Dynamic light-scattering experiments were performed on a laboratory-built goniometer equipped with a diode-pumped laser with single-mode fibre detection and an ALV-5000 correlator as described in the Experimental section. Measurements were performed in PBS (10 mM, pH 7.4) at an HDL concentration of 1 mg of protein/ml and at a scattering angle of 90° at 25°C and a laser power of 200 or 250 mW. Results are the means $\pm$ S.D. for four independent experiments performed in triplicate using different HDL preparations. (B) For the HDL size determination, 12 μg of EL- and LacZ-HDL proteins were analysed by non-denaturing electrophoresis using precast 8–25% gels. After electrophoresis (30 min at 50 V, 30 min at 70 V, 24 h at 150 V), the gels were fixed in sulphosalicylic acid for 5 min and stained with 0.04% Coomassie R 350 for 20 min. Destaining was performed in 20% acetic acid/20% methanol. The HMW (molecular mass) native marker was loaded on to the left of the gel. Results are shown for one experiment out of five, all performed with different EL- and LacZ-HDL preparations.

further whether impaired binding of EL-HDL to SR-BI accounted for the decreased cholesterol efflux, we investigated the binding properties of EL- and LacZ-HDL. Lipoproteins were labelled with Na^{125}I in the protein moiety and incubated with IdIA-7 and IdIA[SR-BI] cells at 37°C for 2 h. As shown in Figure 6, EL-HDL associated less efficiently with IdIA[SR-BI] cells compared with LacZ-HDL. Non-linear regression analysis showed almost identical binding affinity of ^{125}I -LacZ- and ^{125}I -EL-HDL (K_d values, Table 1) and a slightly but significantly ($P < 0.05$) decreased binding capacity (B_{max} , Table 1) of ^{125}I -EL-HDL compared with ^{125}I -LacZ-HDL. Western-blot analysis of EL-HDL using EL-specific antibody [14,15] ruled out the possibility that in the re-isolated HDL after exposure to EL-conditioned media some EL remains physically associated with HDL, which could influence HDL binding to the cells by a bridging function (results not shown). Since it is known that an HDL particle has the ability to promote ABCA1-mediated cholesterol efflux, depending on its ability to act as a donor of lipid-free or lipid-poor pre- β -apoA-I [25,26], we performed a series of experiments on a culture of human monocyte-derived macrophage cell line, THP-1 cells, to examine whether EL modification of HDL alters its ability to mediate ABCA1-dependent cholesterol efflux. THP-cells were

Figure 5 Effect of EL modification on ^3H -cholesterol efflux to HDL

(A) Time course of SR-BI-mediated cholesterol efflux. IdIA-7 and IdIA[SR-BI] cells were labelled with 1 $\mu\text{Ci}/\text{ml}$ ^3H -cholesterol for 30 h. After labelling, the cells were washed twice with Tris-buffered saline containing 0.2% (w/v) BSA and twice with Tris-buffered saline. The cells were then incubated with 100 $\mu\text{g}/\text{ml}$ of EL ($\blacktriangle$)- or LacZ ($\blacksquare$)-HDL in DMEM without FCS. At the indicated time points, aliquots of the medium were collected and counted. (B) Concentration dependence of SR-BI-mediated ^3H -cholesterol efflux. After labelling, IdIA-7 and IdIA[SR-BI] cells were incubated with increasing concentrations of EL ($\blacktriangle$)- and LacZ ($\blacksquare$)-HDL for 2 h. At the end of incubations, the cells were lysed with 0.25 M NaOH to determine the cellular protein content and the cell-associated radioactivity. Total cellular ^3H -cholesterol was calculated as the sum of the radioactivity in the efflux medium plus the radioactivity in the cells and was used to calculate the ^3H -cholesterol efflux (percentage of total ^3H -cholesterol released into the medium). Values obtained with IdIA-7 cells were subtracted from IdIA[SR-BI]. Values in (A) are means $\pm$ S.D. for two independent experiments performed in triplicate. Values in (B) are triplicate determinations from one representative experiment of four, and error bars represent the ranges of the measurements. *** $P \leq 0.001$ (compared with LacZ-HDL); ** $P \leq 0.01$ (compared with LacZ-HDL); * $P \leq 0.05$ (compared with LacZ-HDL).

Figure 6 Cell association of ^{125}I -labelled EL- and LacZ-HDL to SR-BI

IdIA-7 and IdIA[SR-BI] cells were incubated in DMEM at 37°C for 2 h with increasing concentrations of ^{125}I -labelled EL ($\blacktriangle$) and LacZ ($\blacksquare$) HDL. Afterwards cells were washed and lysed with 0.25 M NaOH to determine the cellular protein content and the cell-associated radioactivity. Values obtained with IdIA-7 cells were subtracted from IdIA[SR-BI]. Results are means $\pm$ S.D. for one representative experiment out of three performed in triplicate.

Table 1 Calculation of K_d and B_{max} values by non-linear regression analysis

	K_d ($\mu\text{g/ml}$)	B_{max} (ng/mg)
EL-HDL	38.01 ± 5.33	$664 \pm 32.3^*$
LacZ-HDL	45.02 ± 11.63	863 ± 81.4

* $P < 0.05$ compared with LacZ-HDL.

Figure 7 ABCA1-dependent ^3H -cholesterol (A, B) and ^3H -PL effluxes (C) in THP-1 cells

(A) Cells were plated on to 24-well dishes (250 000/well) and incubated in RPMI + 10% FCS + 160 nM of PMA for 72 h. During the last 24 h, cells were labelled with $1 \mu\text{Ci/ml}$ ^3H -cholesterol and to up-regulate the ABCA1 expression $10 \mu\text{M}$ RA was added to the medium during the last 16 h of incubation. After labelling, cells were washed with PBS + 0.2% BSA

differentiated into macrophages after PMA treatment for 72 h and the expression of ABCA1 was up-regulated by RA without any effect on the expression level of SR-BI as determined by Western-blot analysis (results not shown). To obtain ABCA1-specific cholesterol efflux, parallel experiments were conducted in the presence and absence of 0.8 mM DIDS, an ABC transporter inhibitor. As presented in Figure 7(A), there was no difference between the capacity of EL- and LacZ-HDL to mediate ABCA1-dependent cholesterol efflux, indicating that EL exerts no detectable effect on the capacity of HDL to act as an acceptor for cholesterol effluxed from cells via ABCA1. To rule out the possibility that lipid-free/lipid-poor pre- β apoA-I, whose generation might presumably be facilitated by EL-mediated lipolysis of HDL-PL, was removed during re-isolation of EL- and LacZ-HDL, we used aliquots of the whole incubation mixture as acceptors for cholesterol and PLs in efflux experiments. As shown in Figures 7(B) and 7(C), the capacity of the EL-incubation mixture to mediate ABCA1-dependent cholesterol and PL effluxes was similar to that of the LacZ-incubation mixture.

DISCUSSION

EL is a serum lipase with preferential phospholipase activity and little triacylglycerol lipase activity [11,12]. Studies on EL-ko mice lacking the native murine EL locus as well as in EL-transgenic mice overexpressing the human LIPG locus revealed an inverse relationship between HDL cholesterol plasma level and EL expression [16,17]. Studies *in vitro* showed that EL very efficiently hydrolyses HDL-PL, a major substrate for EL [13–15]. The aim of the present study was to investigate structural and functional properties of HDL modified with EL (EL-HDL) *in vitro*. To obtain EL-HDL, we expressed EL in Cos-7 cells infected with adenovirus encoding human EL and used conditioned medium as a source of EL for the modification of HDL. A similar approach was applied by McCoy et al. [13] to characterize the lipolytic activity of EL. After a 16 h incubation with EL or control medium obtained from Cos-7 cells infected with adenovirus encoding β -galactosidase, HDL was re-isolated by ultracentrifugation and used for structural and functional analyses. Although 1% NEFA-free BSA was present in all incubations, EL-HDL was enriched in NEFA and was consequently more negatively charged than LacZ-HDL. NEFA-free BSA at a concentration of 1% was also used in a previous study where HDL was treated with PLA₂ (phospholipase A₂) to sequester NEFA produced during incubations [7].

and further equilibrated in RPMI 1640 + 0.2% BSA, containing 160 nM PMA and $10 \mu\text{M}$ RA for 2 h. Efflux was performed in the equilibration medium (250 μl /well) either in the presence or absence of 0.8 mM DIDS and indicated concentrations of EL- and LacZ-HDL. After 3 h, media were collected, spun and aliquots were used for scintillation counting. Cells were washed and lysed with 0.25 M NaOH to determine the cell-associated radioactivity and protein content. The efflux was calculated as the percentage of total ^3H -cholesterol released into the medium. Values are means $\pm$ S.D. for a representative experiment performed in triplicate dishes. Key: $\blacktriangle$ —, EL-HDL (+RA); $\blacksquare$ —, LacZ-HDL (+RA); $\triangle$ ---, EL-HDL (+RA/+DIDS); $\square$ ---, LacZ-HDL (+RA/+DIDS). (B) The experiment was performed as in (A) except that whole incubation mixtures containing the indicated amounts of non-re-isolated EL- and LacZ-HDL were used as cholesterol acceptors. Key: $\blacktriangle$ —, EL-HDL (+RA/+DIDS); $\blacksquare$ —, LacZ-HDL (+RA/+DIDS); $\triangle$ ---, EL-HDL (+RA/+DIDS); $\square$ ---, LacZ-HDL (+RA/+DIDS). (C) Cells were labelled with ^3H -choline ($1 \mu\text{Ci/ml}$) for 24 h. After labelling and 2 h equilibration, efflux was performed as described in (B). After 3 h, the efflux medium was collected, spun and extracted by the method of Bligh and Dyer [20]. Aliquots of the organic phase were washed with the methanol/water mixture and used for scintillation counting. Cellular lipids were extracted with hexane/propan-2-ol (3:2, v/v) and dried, subsequently mixed with PBS and finally extracted as described for cell media. Scintillation counting, determination of the protein content and PL efflux calculation were performed as described in (A, B). Key: $\blacktriangle$ —, EL-HDL (+RA/+DIDS); $\blacksquare$ —, LacZ-HDL (+RA/+DIDS); $\triangle$ ---, EL-HDL (+RA/+DIDS); $\square$ ---, LacZ-HDL (+RA/+DIDS). Values in (B) and (C) are the means $\pm$ S.D. for a representative experiment performed in triplicate dishes.

However, in that study, the NEFA abundance and electrophoretic mobility of HDL were not assessed. Sartipy et al. [27] reported that PLA₂ modification of LDL (low-density lipoprotein) resulted in LDL with an increased electrophoretic mobility most probably owing to NEFA bound to LDL, although incubations were performed in the presence of 5% NEFA-free BSA. Similarly, the analysis of the NEFA and lyso-PC abundance in snake venom PLA₂-treated HDL revealed that a certain amount of NEFA and lyso-PC cannot be removed from the treated HDL even in the presence of a large amount of NEFA-free BSA [28]. Besides NEFA, lyso-PC, another cleavage product of EL-mediated HDL-PC hydrolysis, accumulated in EL-HDL. Although the PC content of EL-HDL was decreased by 40%, the size of EL-HDL was unaltered and was not significantly different from that of LacZ-HDL. In contrast, small HDL particles, severely depleted in PL, FC and CE, were generated in mice transduced with an EL adenovirus [29]. Generation of small HDL particles and also pre- β -HDL in these mice might be explained by much higher EL activities and, consequently, more severe HDL-PC depletion achieved after transduction *in vivo* compared with the HDL modification conditions *in vitro*. Additionally, a net depletion of CE, most probably by selective CE uptake via SR-BI [29,30] might promote destabilization of the PC-depleted HDL and concomitantly dissociation of lipid-free or lipid-poor apoA-I [31]. To assess the possibility that the lyso-PC, which are retained in EL-HDL, are responsible for the unaltered particle size, we modified HDL with EL in the presence of 5% NEFA-free BSA. After incubation, EL-HDL contained markedly decreased amounts of NEFA and undetectable amounts of lyso-PC and was similar in size compared with control LacZ-HDL (results not shown), ruling out the possibility that the NEFA and lyso-PC that remain in HDL after modification are responsible for the unaltered size of EL-HDL. Previous studies have shown that the efficiency of cholesterol efflux was strongly correlated to HDL-PC content [7,32–34]. Provided that the PL layer on the HDL surface serves as a region that can efficiently solubilize cholesterol, it is conceivable that the depletion of HDL-PC leads to an insufficient solubilization of cholesterol molecules desorbed from the cells, resulting in a decreased rate of SR-BI-dependent cholesterol efflux. To test the effect of EL modification on the capacity of HDL to mediate removal of FC from cells, we performed ³H-cholesterol efflux experiments. EL-HDL compared with LacZ-HDL showed a decreased ability to mediate ³H-cholesterol efflux from IdIA-7 cells expressing minimal levels of SR-BI [5,10] and this effect was more pronounced in SR-BI overexpressing IdIA[SR-BI] cells. Interestingly, cholesterol efflux to EL-HDL was significantly impaired only at concentrations of 100 μ g/ml HDL and above, which is in sharp contrast with a finding demonstrating a decreased cholesterol efflux to PLA₂-HDL (PLA₂-modified HDL) also at concentrations of 20 μ g/ml HDL [7]. This difference between EL- and PLA₂-HDL may be explained by the different rates of PC depletion after treatment with purified PLA₂- and EL-conditioned medium (79% versus 40% reduction respectively). To assess whether the 40% depletion in the PC content might be insufficient to impact the ability of EL-HDL to mediate cholesterol efflux at lower HDL concentrations, we modified HDL with higher EL activities yielding a 55% depletion in the HDL-PC content. Cholesterol efflux mediated by EL-HDL was profoundly diminished at concentrations of 100 and 200 μ g/ml HDL compared with LacZ-HDL. At both low HDL concentrations, 20 and 50 μ g/ml respectively, the EL-HDL-mediated cholesterol efflux was impaired, however, this was not statistically significant compared with LacZ-HDL (results not shown). It is possible that more severe depletion (more than 55%) in the PC content which we failed to achieve in our experimental system is required to

obtain significantly impaired cholesterol efflux at lower HDL concentrations. Previous studies demonstrated that apoA-I conformation, which is responsible for the nature and concentration of HDL lipids including HDL-PC [8,31], markedly impairs interaction of HDL with SR-BI [9], which in turn might impair cholesterol efflux from cells [10]. Despite profoundly altered HDL composition after EL modification, especially owing to a PC depletion together with the enrichment in NEFA and lyso-PC, the binding affinity of HDL to SR-BI remained almost unaltered after EL modification. In contrast with the nearly unaltered binding affinity, we found slightly but significantly decreased binding capacity ($B_{\max}$ values) of EL-HDL to SR-BI compared with LacZ-HDL which might explain, at least in part, and in combination with the PC depletion, why changes in SR-BI-mediated cholesterol efflux between EL- and LacZ-HDL could be observed only at higher (100 μ g/ml and above) lipoprotein concentrations. In contrast with the unaltered binding affinity of EL-HDL, the binding affinity of PLA₂-HDL [7] was decreased by 2-fold whereas its binding capacity remained unaltered. These differences, apart from the different rates of PC depletion, might be ascribed to the different cell-culture systems used for binding and efflux studies. Additionally, different binding properties and different capacity of EL- and PLA₂-HDL [7] to mediate SR-BI-dependent cholesterol efflux might also be explained by the difference in their lyso-PC and NEFA composition due to the sn1-specific EL and sn2-specific PLA₂ cleavages of HDL-PC. We found that *in vitro* EL modification of HDL exerts no detectable effect on the ability of HDL to mediate ABCA1-dependent cholesterol and PL effluxes. These findings are in sharp contrast with the findings of a most recent study [36] demonstrating that the serum obtained from apoA-I transgenic mice overexpressing EL after adenoviral transduction exhibits, in addition to a decreased ability to mediate SR-BI-dependent cholesterol efflux, which is consistent with our results, an increased capacity to mediate ABCA1-dependent cholesterol efflux. This discrepancy between our results and those reported by Yancey et al. [36] might be explained by the fact that, after EL overexpression, HDL is severely depleted in PC that has as a consequence the accelerated selective uptake of HDL-CE via SR-BI [30], concomitant depletion of the HDL core and subsequent destabilization of the particle accompanied by the dissociation of lipid-free or lipid-poor apoA-I [37] acting as an acceptor for cholesterol effluxed via ABCA1. An additional pool of the lipid-free or lipid-poor apoA-I in the apoA-I transgenic/EL overexpressing mice might arise from the newly synthesized free apoA-I and nascent HDL whose lipidation and maturation are affected by the action of EL [29]. Because the HDL-PC depletion is not sufficient for HDL remodelling [31], and *in vitro* incubation reactions lack the SR-BI and CE transfer protein required for the depletion of the HDL core lipids and concomitant remodelling of HDL and dissociation of apoA-I [31,37], it is not surprising that the modification of HDL with EL *in vitro* has no detectable effect on ABCA1-dependent cholesterol and PL effluxes.

Taken together, our results demonstrate that the *in vitro* EL modification of HDL alters the chemical composition and physical properties of HDL resulting in the slightly but significantly decreased binding capacity of HDL to SR-BI-overexpressing cells and a markedly diminished ability of HDL to mediate SR-BI-dependent cholesterol efflux at high lipoprotein concentrations. EL modification of HDL had no impact on the ability of HDL to mediate ABCA1-dependent cholesterol and PL effluxes.

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