A high-density-lipoprotein receptor appears to mediate the transfer of essential fatty acids from high-density lipoprotein to lymphocytes

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It has been shown previously that a specific high-density lipoprotein (HDL) receptor exists on human lymphocytes that recognizes apoprotein (apo) A1 as its ligand, and may be responsible for utilization of HDL lipids to respond optimally to mitogenic stimulation when cultured in serum-free medium supplemented with HDL. To clarify further the relationship between various HDL lipids used by lymphocytes and HDL receptor activity, the lipid composition of the cells and the regulation of HDL and low-density lipoprotein (LDL) receptors on freshly isolated lymphocytes and mitogen-activated T-blasts after treatment with lipoproteins, liposomes or fatty acids were investigated. Our data show that the linear increase in cell proliferation correlates with the presence of HDL in fatty-acid-free culture medium in the concentration range of HDL receptor saturation. Decreased binding/uptake of dioctadecylindocarbocyanine (DiI)-fluorescence-labelled HDL by freshly isolated lymphocytes was observed in the presence of unlabelled HDL in 4-day culture, whereas T-blast binding/uptake was down-regulated by preincubation not only with HDL but also with LDL. T-blasts pretreated with HDL showed increased cellular contents of phosphatidylcholine, oleic acid ($C_{18:1,n-9}$) and linoleic acid ($C_{18:2,n-6}$), which are essential for optimal proliferation of mitogen-stimulated lymphocytes. Furthermore, DiI-HDL binding on lymphocytes was down-regulated by up to 20% (resting T cells) and 50% (T-blasts) when cultured in the presence of apoA1-phosphatidylcholine liposomes (T-blasts only), oleic acid or linoleic acid, but not by stearic acid ($C_{18:0}$). The results indicate that HDL provide lymphocytes with essential fatty acids, which in turn regulate HDL receptor activity.

INTRODUCTION

Since Glomset [1] hypothesized that high-density lipoprotein (HDL) may deliver cholesterol from peripheral cells to the liver, HDL interactions with cells have been widely investigated. The existence of an HDL receptor on various types of cells, e.g. fibroblasts, arterial smooth-muscle cells [2], aortic endothelial cells [3] and peritoneal macrophages [4,5], has been described by several groups. The primary effect of HDL receptors on these cells may involve reverse excess cholesterol transport [2,4,6], and HDL receptor activity is regulated by the cellular cholesterol content [6]. However, direct demonstration *in vivo* and quantification of reverse cholesterol transport and receptor regulation in experimental animals or humans remain to be done [7].

During the last decade, the possible role of HDL for the preservation of cellular cholesterol homeostasis has been studied extensively. However, very little is known about the relationship of HDL to cellular phospholipids and fatty acids, although HDL contains 22% phospholipids and 3% non-esterified fatty acids. Our previous studies demonstrated the existence of an HDL-specific receptor on human peripheral-blood lymphocytes (PBL) that recognizes apoprotein (apo) A1 as its ligand [8,9]. This binding site is responsible for lipid binding/uptake, and concomitant internalization and degradation of apoproteins from HDL, but we found no evidence of HDL-mediated reverse cell

membrane cholesterol transport in lymphocytes [10]. *In vitro*, HDL can rescue mitogen [phytohaemagglutinin (PHA)]-stimulated lymphocyte proliferation suppressed by mevinolin, a hydroxymethylglutaryl-CoA reductase inhibitor. HDL also promotes cell proliferation in lipid-free tissue-culture medium [9], and thus seems able to provide lipids to proliferating lymphocytes.

To identify the type of lipids supplied by HDL via its specific receptor, we investigated the relationship between lymphocyte lipid composition and regulation of HDL receptor activity on freshly isolated PBL and PHA-stimulated T-blasts.

MATERIALS AND METHODS

Isolation and labelling of lipoproteins

EDTA-treated plasma was pooled from normolipemic fasting (12–14 h) male and female donors (20–35 years) with very low lipoprotein(a) levels (< 1 mg/dl). Lipoproteins were prepared by differential centrifugation, solid KBr being used to adjust the density. The following fractions were obtained: low-density lipoprotein (LDL), 1.020–1.050 g/ml; HDL₂, 1.125–1.230 g/ml. HDL₂ was purified twice further on heparin–Sepharose affinity columns (Pharmacia Fine Chemicals AB, Uppsala, Sweden) to remove the apoE-containing fraction. Lipoprotein concentrations were determined gravimetrically by weighing a sample after

Abbreviations used: apo, apoprotein(s); DiI, 3',3'-dioctadecylindocarbocyanine perchlorate; FACS, fluorescence-activated cell sorter; FI, fluorescence intensity; HDL, high-density lipoprotein(s); LDL, low-density lipoprotein(s); PBL, peripheral-blood lymphocytes; PBS, phosphate-buffered saline; PHA, phytohaemagglutinin; UdR, iodo-2-deoxyuridine.

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drying; lipoprotein quantities are expressed in terms of their total weights.

Lipoproteins were fluorescence-labelled with 3',3'-dioctadecyl-indocarbocyanine perchlorate (DiI; excitation 520 nm, emission 570 nm; Lambda Probes and Diagnostics, Graz, Austria) in accordance with Pitas *et al.* [11], with the following modifications: 26 mg of HDL was incubated in 50 ml of lipoprotein-deficient plasma with 980 µg of DiI/ml solubilized in 650 µl of dimethyl sulphoxide for 8 h at 37 °C. After adjustment of the density to 1.225 g/ml with KBr, DiI-HDL was re-isolated by ultracentrifugation [9].

The electrophoretic mobility of labelled LDL and HDL was compared on agarose gels (Lipidophor System; Immuno AG, Vienna, Austria) with that of unlabelled preparations. Chloramphenicol (50 mg/l; Serva, Heidelberg, Germany), kallikrein inactivator (Trasyol, 100 000 units/l; Bayer, Leverkusen, Germany) and EDTA (1 g/l; Merck, Darmstadt, Germany) were present during all steps of lipoprotein preparation and labelling to prevent lipid peroxidation and apoB cleavage by contaminating bacterial or serum proteinases.

Isolation and cultivation of PBL

Mononuclear cells were isolated from fresh anti-coagulated peripheral blood of healthy blood donors of both sexes, aged 20–30 years, by density-gradient centrifugation over Lymphopaque (Nyegaard and Co., Oslo, Norway; density 1.086 g/l) with monocytes depleted by adherence to plastic in tissue-culture flasks, as described [8,9].

PBL cultivation was performed as previously described [8–10,12]. Briefly, serum-free Iscove's modified Dulbecco's medium (Gibco, Paisley, Scotland, U.K.) was supplemented with 0.5% BSA (fatty acid-free; Sigma, Munich, Germany), monothioglycerol (Sigma; 10 µg/ml) and human transferrin (Sigma; 1 µg/ml). Mitogen responses were assayed in triplicate in round-bottomed 96-well tissue-culture plates (Nunc, Roskilde, Denmark) in medium with or without the indicated lipoprotein or fatty acids in a final volume of 200 µl. PBL (2.5×10^5 /well) were stimulated with 12.5 µg of PHA/ml (Difco, Detroit, MI, U.S.A.). Stimulation was assessed by incorporation of 5-[¹²⁵I]iodo-2-deoxyuridine (¹²⁵I-UdR; Amersham International, Amersham, Bucks., U.K.), and 0.1 µCi/well was added with 0.1 µM-fluorodeoxyuridine (Sigma) during a 3 h pulse on day 4 of culture. T lymphocytes for lipid analysis and lipoprotein binding/uptake studies were cultivated with or without PHA at 37 °C for 4 days in 50 ml Falcon tissue-culture flasks (Becton Dickinson and Co., Oxnard, CA, U.S.A.) in a final volume of 4 ml with or without the indicated concentrations of apoE-free HDL, LDL, apo A1–L-α-phosphatidylcholine liposomes, and oleic acid (C_{18:1,n-9}), linoleic acid (C_{18:2,n-6}) or stearic acid (C_{18:0}) (free acids; Sigma). Human apo A1 was isolated from HDL, and L-α-phosphatidylcholine was purchased from Sigma. Details of methods of apo A1 liposome preparation are described elsewhere [13]. Non-esterified fatty acids were dissolved at a concentration of 50 mM in ethanol and added directly to cultures [14]. An equivalent volume of ethanol only (final concn. 0.1%, v/v) was added to control cultures and found to have no significant effect on proliferation and HDL or LDL binding/uptake of lymphocytes.

Lipid analyses

PBL were cultivated with or without PHA (12.5 µg/ml) in serum-free medium with or without apoE-free HDL or LDL (50 µg/ml) for 4 days at 37 °C. After four washes with phosphate-buffered saline (PBS; 0.01 M-Na₂HPO₄, 0.003 M-KH₂PO₄, 0.116 M-NaCl, pH 7.2), cellular lipids were extracted with

chloroform/methanol (1:1, v/v) containing 0.01% butylhydroxytoluene at room temperature. Cholesterol and phospholipids were fractionated by t.l.c. using a Camag-Linomat III instrument (Camag AG, Dürren, Germany), as described previously [12]. Commercial standards (cholesterol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine; all from Sigma) were used for chromatographic quantification. Samples of lipid extracts were methylated and separated into total fatty acids by g.l.c. (Dani-6500; Kontron, Zürich, Switzerland) as described previously [12]. C₁₇ methyl ester was used as an internal standard.

LDL and HDL binding/uptake studies

Lipoproteins were extensively dialysed against Iscove's medium before use. After three washes of treated cells, binding/uptake studies were performed by incubating T-cells for 2 h at 37 °C with DiI-HDL or DiI-LDL (25 µg/ml) in Iscove's medium containing 0.5% BSA. After five further washes with PBS, fluorescence measurements were performed with a fluorescence-activated cell sorter (FACS III; Becton Dickinson, Sunnyvale, CA, U.S.A.) equipped with an argon ion laser (model 2025; Spectra Physics, Mountain View, CA, U.S.A.) and linked to an Apple II Plus computer. Details of FACS settings and methods of quantification of fluorescence intensity (FI) for uptake studies have been described elsewhere [8–10,12,15,16]. Briefly, the conditions were as follows: laser excitation, 514.5 nm, 0.6 W output power; optical filters, 520 nm interference long pass and 530 nm 'cut-on' colour glass, photomultiplier-tube settings 440–740 V, depending on the FI. For each sample 10⁴ cells were analysed; unlabelled cells were included as a zero reference to control for the signal/noise ratio at each photomultiplier-tube setting. FI was quantified by determining the FI of the 50th percentiles from the cumulative frequency.

Other analytical procedures

Protein contents of samples were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). Statistical analyses were performed by an unpaired Student's *t* test.

RESULTS

Regulation of HDL receptor activity and enhancement of cell proliferation by HDL and LDL

We have previously shown the existence of a HDL receptor on human lymphocytes and hypothesized its involvement in providing lipids to growing T cells [9]. To assess the effects of HDL and LDL on their respective receptor activity and on cell proliferation, PBL were cultivated with or without PHA in fatty-acid-free medium supplemented with defined concentrations of apoE-free HDL or LDL for 4 days. Increasing concentrations of unlabelled HDL in freshly isolated PBL culture showed that binding/uptake of DiI-HDL decreased to 25% of controls. DiI-LDL binding also showed a slight decrease rather than the expected up-regulation of LDL receptor expression (Fig. 1a). After 4 days preincubation with the indicated concentrations of LDL, its receptor activity was down-regulated to less than 10% of control, whereas DiI-HDL binding totalled about 75% of control (Fig. 1b). This decreased DiI-HDL binding cannot be explained by binding via the apoB_E and scavenger receptors, since EDTA was present during DiI-HDL binding assays although apoE-free HDL₃ was used.

Cell proliferation was enhanced and reached a plateau at a concentration of 50–120 µg/ml with increasing concentrations of both HDL or LDL (Fig. 2). The effects of supplementing the

Fig. 1. Down-regulation of DiI-HDL binding/uptake of human lymphocytes by lipoproteins

PBL (2×10^6) were cultivated in serum-free medium supplemented with the indicated concentrations of apoE-free HDL (a) or LDL (b) at 37 °C for 4 days. The cells were then washed extensively, re-incubated for 2 h at 37 °C in Iscove's medium to minimize lipoprotein binding to the cell surface and labelled for 2 h at 37 °C with 25 μg of DiI-HDL/ml (○) plus EDTA (12.5 mM) or DiI-LDL (●) without EDTA in Iscove's medium containing 0.5% BSA. DiI FI is expressed as percentage of control (FI 50 percentile: 15–22 FACS channels) in serum-free medium. Data are means of three experiments.

culture medium with lipoprotein on the subsequent DiI-HDL and DiI-LDL binding/uptake by day 4 PHA-activated blasts are also shown in Fig. 2. HDL down-regulates not only its own receptor, but also, albeit to a lower extent, the LDL receptor, and vice versa. Both receptor activities seem to be controlled in parallel, depending on the cell requirement for lipids. On the other hand, T-blasts express measurable numbers of HDL and LDL receptors even in the presence of relatively high, but still physiological, HDL or LDL concentrations, reflecting their much greater requirements for lipids during proliferation.

Effect of HDL and LDL on lipid composition of lymphocytes

The observation that HDL and LDL supplementation of culture medium induces down-regulation of the activity of both receptors led us to investigate further possible changes of cellular lipid composition in T lymphocytes after preincubation with

Fig. 2. Effect of lipoproteins on proliferation and DiI-HDL binding/uptake by T lymphocytes

To determine cell proliferation, PBL (2.5×10^5 /well) were cultivated in a 96-well plate with PHA (12.5 $\mu\text{g/ml}$) and apoE-free HDL or LDL for 4 days at 37 °C. T-blasts were pulsed with ${}^{125}\text{I-UdR}$ (0.1 $\mu\text{Ci/well}$) at 37 °C for the last 3 h and then harvested to measure cell-associated radioactivity (▲). To assess DiI-HDL and -LDL binding, PBL (2.5×10^6 /ml) were activated with PHA in serum-free medium supplemented with the indicated concentrations of apoE-free HDL (a) or LDL (b) at 37 °C for 4 days. T-blasts were then washed and re-incubated for 2 h at 37 °C in medium alone, and labelled for 2 h at 37 °C with 25 μg of DiI-HDL/ml (○) plus EDTA (12.5 mM) or DiI-LDL (●) without EDTA. FI of the labelled cells was measured by FACS. The FI 50 percentile of control cells lies between 31 and 45 FACS channels. Values are means of two experiments.

HDL or LDL. By using the same experimental procedures as described above, PBL were cultivated in the absence or presence of 50 μg of HDL or LDL/ml with or without PHA, and cellular lipids were determined after extraction with methanol/chloroform. PBL preincubated with HDL showed a slightly increased cholesterol content, although no significant difference could be seen compared with that of control cells incubated in medium only. On the other hand, the cholesterol level of the cells preincubated with LDL was significantly higher (29.74 ± 7.98 versus 16.57 ± 6.27 ; $P < 0.05$) than that of control lymphocytes (Table 1). The results of cellular fatty acid analyses, summarized in Table 1, show that the linoleic acid ($\text{C}_{18:2,n-6}$) content of HDL-incubated cells was significantly increased compared with that of control T cells (54.58 ± 11.34 versus 32.00 ± 6.20 ; $P < 0.05$), and

Table 1. Effect of lipoproteins on lipid composition of resting T cells

PBL (2.5×10^6 /ml) were incubated in serum-free medium supplemented with or without apoE-free HDL or LDL ($50 \mu\text{g}/\text{ml}$) for 4 days at 37°C . After four washes with PBS the cellular lipids were extracted with methanol/chloroform (1:1, v/v). Cholesterol and phospholipids were analysed by t.l.c. Fatty acids were determined by g.l.c. Values are means $\pm$ s.e.m. from four blood donors: *significantly different from control ($P < 0.05$).

Lipid	Lipid content ($\mu\text{g}/\text{mg}$ of cell protein)		
	T cells (control)	T cells preincubated with LDL	T cells preincubated with HDL
Total cholesterol	16.57 ± 6.27	$29.74 \pm 7.98^*$	21.98 ± 5.9
Phosphatidylcholine	34.58 ± 19.37	39.81 ± 18.25	40.68 ± 15.27
Phosphatidylethanolamine	29.75 ± 13.98	31.66 ± 9.55	34.77 ± 5.21
Phosphatidylserine	6.87 ± 1.23	5.7 ± 1.68	5.52 ± 1.03
Total phospholipids	57.09 ± 11.35	66.91 ± 11.19	69.34 ± 7.47
Fatty acid (nmol/mg of cell protein)			
C _{16:0}	120.48 ± 27.73	119.76 ± 12.3	127.75 ± 22.32
C _{16:1}	2.92 ± 0.56	2.35 ± 0.71	2.58 ± 0.77
C _{18:0}	93.66 ± 13.48	90.56 ± 17.88	89.86 ± 10.56
C _{18:1, n-7}	10.71 ± 1.27	10.17 ± 2.86	15.27 ± 3.10
C _{18:1, n-9}	55.16 ± 16.58	54.70 ± 12.7	62.19 ± 19.56
C _{18:2, n-6}	32.00 ± 6.2	44.72 ± 15.86	$54.58 \pm 11.34^*$
C _{20:0}	2.38 ± 0.55	2.25 ± 0.41	2.84 ± 0.14
C _{20:1, n-9}	3.87 ± 1.30	3.82 ± 1.22	4.53 ± 1.12
C _{20:2, n-6}	5.91 ± 1.24	5.28 ± 1.72	5.89 ± 0.52
C _{20:3, n-6}	9.87 ± 2.38	12.15 ± 1.85	9.69 ± 1.29
C _{20:4, n-6}	36.68 ± 8.13	44.38 ± 4.98	50.43 ± 19.83
C _{22:0}	4.26 ± 0.40	3.96 ± 0.78	5.20 ± 0.67
C _{22:4, n-6}	10.88 ± 1.86	9.83 ± 2.97	10.77 ± 1.09
C _{22:5, n-3}	8.98 ± 1.25	8.01 ± 1.46	10.45 ± 3.27
C _{22:6, n-3}	9.25 ± 1.40	10.38 ± 6.14	11.75 ± 1.20
C _{24:0}	2.82 ± 0.65	2.21 ± 0.43	2.57 ± 0.88
C _{24:1}	3.07 ± 0.76	2.51 ± 0.90	3.55 ± 1.09

Table 2. Effect of lipoproteins on lipid composition of T-blasts

PBL (2.5×10^6 /ml) were incubated with PHA ($12.5 \mu\text{g}/\text{ml}$) in serum-free medium supplemented with or without apoE-free HDL or LDL ($50 \mu\text{g}/\text{ml}$) for 4 days at 37°C . The procedure of lipid analysis was same as described in Table 1. Values are means $\pm$ s.e.m. from four blood donors: *significantly different from control; †significantly different from T-blasts preincubated with LDL ($P < 0.05$).

Lipid	Lipid content ($\mu\text{g}/\text{mg}$ of cell protein)		
	T-blasts (control)	T-blasts preincubated with LDL	T-blasts preincubated with HDL
Total cholesterol	35.13 ± 14.26	$62.60 \pm 15.54^*$	49.3 ± 16.11
Phosphatidylcholine	45.51 ± 25.22	56.48 ± 15.17	$87.06 \pm 8.46^{*†}$
Phosphatidylethanolamine	48.14 ± 23.07	44.58 ± 17.58	60.75 ± 14.44
Phosphatidylserine	11.61 ± 6.58	16.9 ± 5.34	18.14 ± 2.6
Total phospholipids	109.06 ± 61.35	130.49 ± 34.99	181.55 ± 15.16
Fatty acid (nmol/mg of cell protein)			
C _{16:0}	128.15 ± 35.97	188.83 ± 77.44	$235.10 \pm 64.42^*$
C _{16:1}	25.36 ± 16.29	19.61 ± 7.6	25.79 ± 6.78
C _{18:0}	84.37 ± 40.65	82.73 ± 17.23	126.24 ± 28.42
C _{18:1, n-7}	41.80 ± 23.87	38.21 ± 13.17	51.33 ± 16.58
C _{18:1, n-9}	88.29 ± 28.50	98.38 ± 30.2	$156.23 \pm 20.05^{*†}$
C _{18:2, n-6}	25.48 ± 10.18	$60.64 \pm 19.71^*$	$76.22 \pm 17.22^*$
C _{18:3, n-3}	5.49 ± 0.27	8.05 ± 1.35	5.22 ± 2.35
C _{20:0}	2.11 ± 0.87	3.03 ± 2.37	3.06 ± 0.22
C _{20:1, n-9}	6.49 ± 3.22	6.37 ± 1.39	8.96 ± 1.42
C _{20:2, n-6}	2.29 ± 1.62	3.79 ± 1.09	5.38 ± 1.16
C _{20:3, n-6}	8.38 ± 5.14	12.11 ± 3.18	$20.72 \pm 4.96^{*†}$
C _{20:4, n-6}	44.47 ± 20.87	43.30 ± 9.85	65.33 ± 14.64
C _{22:0}	5.73 ± 1.69	5.35 ± 2.16	6.19 ± 1.27
C _{22:4, n-6}	8.21 ± 2.73	7.49 ± 2.49	8.86 ± 1.76
C _{22:5, n-3}	5.82 ± 2.50	6.21 ± 2.62	7.56 ± 1.52
C _{22:6, n-3}	4.69 ± 2.66	4.72 ± 1.22	8.71 ± 1.61
C _{24:0}	4.08 ± 1.75	3.72 ± 1.20	4.61 ± 0.27
C _{24:1}	3.79 ± 1.26	4.04 ± 1.00	5.06 ± 0.63

that the levels of oleic acid (C_{18:1, n-9}) and arachidonic acid (C_{20:4, n-6}) were also increased [62.19 ± 19.56 versus 55.16 ± 16.58 ($P = 0.19$) and 50.43 ± 19.83 versus 36.68 ± 8.13 ($P = 0.11$)] although not to a statistically significant extent. These results led to the assumption that the main function of HDL in this system may be to provide fatty acids, especially essential fatty acids, to lymphocytes to maintain cellular lipid homeostasis.

Mitogen-activated T-blasts pretreated with HDL or LDL also revealed a difference in lipid composition. The results of cholesterol, phospholipid and fatty acid analyses on T-blasts are summarized in Table 2. Incubation of T-blasts with LDL significantly increased total cellular cholesterol (62.60 ± 15.54 versus 35.13 ± 14.26 , $P < 0.05$) and linoleic acid (C_{18:2, n-6}; 60.64 ± 19.71 versus 25.48 ± 10.18 , $P < 0.05$). Phosphatidylcholine and palmitic acid (C_{16:0}) were also present in higher levels compared with controls, although no significant difference emerged (188.83 ± 77.44 versus 128.15 ± 35.97 , $P = 0.09$). After preincubation of T-blasts with HDL, the contents of cellular phosphatidylcholine (87.06 ± 8.46 versus 45.51 ± 25.22), oleic acid (156.23 ± 20.05 versus 88.29 ± 28.50) and homo- γ -linolenic acid (C_{20:3, n-6}; 20.72 ± 4.96 versus 8.38 ± 5.14) were significantly higher than that of T-blasts ($P < 0.05$), and even of cells preincubated with LDL. Palmitic acid and linoleic acid in these cells were also significantly increased compared with

T-blasts pre-cultured in medium alone (235.10 ± 64.42 versus 128.15 ± 35.97 and 76.22 ± 17.22 versus 25.48 ± 10.18 respectively; $P < 0.05$). The results indicate that HDL mainly supply fatty acids and/or phospholipids to proliferating T-blasts, whereas LDL mainly deliver cholesterol.

No evidence for HDL-mediated reverse cholesterol transport from T-blasts

As shown in Table 2, the cellular cholesterol content was significantly higher in LDL-treated T-blasts than control cells cultivated in Iscove's medium only, whereas HDL-treated cells showed no decrease in their cholesterol. To assess a possible HDL-mediated reverse excess-cholesterol transport, PBL were cultivated with PHA in the presence of LDL ($50 \mu\text{g}/\text{ml}$) for 3 days and then re-incubated with HDL ($50 \mu\text{g}/\text{ml}$) for another day after extensive washing with medium. Analyses of cellular cholesterol showed that T-blasts cultured for 3 days with LDL had significantly increased cellular cholesterol levels compared with control (43.28 ± 3.55 versus $33.62 \pm 2.46 \mu\text{g}/\text{mg}$ of cell protein; $P < 0.01$), and these levels were not decreased by a further 1 day of culture with HDL at 37°C (43.28 ± 3.55 versus $41.16 \pm 3.40 \mu\text{g}/\text{mg}$ of cell protein). These results suggest that HDL are not capable of reverse transport of cellular cholesterol from T-blasts.

Table 3. Regulation of DiI-HDL binding/uptake by apoA1 liposomes on day 4 T-blasts

PBL were incubated for 4 days at 37 °C with PHA (12.5 µg/ml) in serum-free medium supplemented with the indicated concentrations of apoA1 liposomes. After extensive washing, the T-blasts were re-incubated for 2 h at 37 °C, and labelled for 2 h at 37 °C with 25 µg of DiI-HDL/ml plus EDTA (12.5 mM) or DiI-LDL without EDTA. FI of the cells was determined by FACS analysis after four washes with PBS. The FI 50 percentile of control cells lies between 31 and 45 FACS channels. Values are means of duplicate experiments.

ApoA1 liposome (µg/ml)	Binding/uptake (% of control)	
	DiI-HDL	DiI-LDL
3.25	85	100
7.5	69	104
15	38	112
30	35	120
60	38	134

Fig. 3. Regulation of DiI-HDL binding/uptake by fatty acids on resting T cells

PBL (2.5×10^6 /ml) were incubated for 4 days at 37 °C in serum-free medium (□) supplemented with 10 µM-oleic acid (▨), -linoleic acid (▤) or -stearic acid (■) solubilized in ethanol (50 mM). T cells were extensively washed and labelled for 2 h at 37 °C with 25 µg of DiI-HDL/ml plus EDTA (12.5 mM) or DiI-LDL (b) without EDTA. Cell-associated FI was determined by FACS analysis after four washes. The FI 50 percentile of control cells lies between 31 and 45 channels. Values are means $\pm$ S.D. ($n = 4$); * $P < 0.05$.

Essential fatty acids regulate HDL receptor activity

To evaluate the possible role of phosphatidylcholine and essential fatty acids in the regulation of HDL and LDL receptor activity, PBL were cultured with or without PHA in fatty-acid-free medium supplemented with apoA1 liposomes or fatty acids. The representative experiment shown in Table 3 indicates that DiI-HDL binding/uptake was down-regulated to 38 %, whereas that of DiI-LDL was up-regulated to 134 % after incubation in the presence of apoA1 liposomes (60 µg/ml). Interestingly, resting T cells treated with oleic acid or linoleic acid exhibited a significant ($P < 0.05$) down-regulation of DiI-HDL binding/uptake (Fig. 3a), and a considerably less pronounced effect on DiI-LDL binding (Fig. 3b). The presence of stearic acid ($C_{18:0}$), a saturated fatty acid, in the culture medium did not affect either DiI-HDL or -LDL binding/uptake (Fig. 3).

Fig. 4. Effect of fatty acids on proliferation and DiI-HDL binding of T lymphocytes

Oleic acid (a), linoleic acid (b) or stearic acid (c) solubilized in ethanol (50 mM) at the indicated concentrations was added to the cells in round-bottomed microtitre plates in a final volume of 0.2 ml of fatty-acid-free medium containing 2.5×10^6 PBL stimulated with 12.5 µg of PHA/ml. After 4 days of culture at 37 °C, lymphocyte proliferation was quantified by determining 125 I-UdR incorporation (Δ). In parallel, PBL (1×10^7) were cultivated in 4 ml of the medium supplemented with the fatty acids and PHA as above. T-blasts were labelled with DiI-HDL (□) or -LDL (■; 25 µg/ml) for 2 h at 37 °C. Cell-associated FI was measured after 4 washes. The FI 50 percentile of control cells lies between 31 and 45 channels. Values are means $\pm$ S.E.M. ($n = 4$); * $P < 0.01$.

In mitogen-stimulated T-cell cultures, supplementation with oleic acid (Fig. 4a) or linoleic acid (Fig. 4b) increased UdR incorporation by the cells 4-fold (5000 to 22000 c.p.m.) to a plateau at a concentration of 5 µM, whereas stearic acid did not significantly enhance cell proliferation (Fig. 4c). These results imply the importance of essential fatty acids for optimal proliferation of PHA-activated T cells. Simultaneously, treatment of T-blasts with oleic acid or linoleic acid evoked a 50 % down-

regulation of DiI-HDL binding/uptake and had no effect on DiI-LDL binding (Figs. 4*a* and 4*b*). DiI-HDL or -LDL binding by T-blasts was obviously not regulated by preincubation with stearic acid (Fig. 4*c*), comparable with the situation in resting T cells treated with fatty acids. These data suggest that HDL receptor activity on human lymphocytes may be regulated by cellular phospholipids or essential fatty acids rather than cellular cholesterol.

DISCUSSION

On the basis of the very strong negative correlation between blood HDL levels and coronary heart disease risk, a great deal of evidence *in vitro* concerning the protective mechanism of HDL has been reported, of which the most attractive hypothesis certainly is the reverse cholesterol transport from peripheral tissues to the liver [1,6,17]. However, direct demonstration *in vivo* and quantification of cholesterol reverse transport in either experimental animals or man is still lacking. Alternative hypotheses suggest lipid supplementation by HDL to various types of cells; for example, HDL-associated cholesteryl ester can be selectively taken up by macrophages [5] and steroidogenic tissues [18–20]. HDL might also provide lipids to endothelial cells [21,22], virus-transformed lymphoblastoid cells [23,24] and brain tissue [25]. For human lymphocytes, we have shown that PHA-cultured T-cell proliferation suppressed by mevinolin, an inhibitor of hydroxymethylglutaryl-CoA reductase, can be rescued by HDL in high (above receptor saturation), albeit still physiological, concentrations [9], and holo-HDL particles may be internalized and degraded by T-blasts [10], as suggested in a recent review on lipoprotein interaction with T cells [26]. In the present study, preincubation of PBL with apo E-free HDL led to cellular cholesterol levels higher than, or at least equal to, those of controls, although no statistical significance was noted. Our data do not support the existence of an HDL-mediated reverse cholesterol-transport mechanism in human lymphocytes, as emphasized by the fact that LDL receptor activity on T-blasts was down-regulated by HDL preincubation; e.g. had an HDL-mediated reverse cholesterol transport from T cells occurred during incubation, increased expression of LDL receptor should have been seen, since the activity of this latter receptor is sensitively regulated by the cellular cholesterol content [27]. Furthermore, the elevated cellular cholesterol content of T-blasts loaded with LDL was not normalized to a level similar to that of controls by 24 h incubation with HDL, indicating that HDL cannot reverse-transport excess cholesterol from T cells in this system. Therefore the physiological functions of HDL do not only include reverse cholesterol transport.

The view that HDL receptor activity is mainly regulated by the cellular cholesterol content is also challenged by the fact that decreased HDL receptor numbers on enterocytes were observed without a change of cellular cholesterol content when endogenous cholesterol synthesis of these cells was inhibited by lovastatin (equal to mevinolin [28]).

Recently, a proliferation-promoting effect of HDL on various types of cells was described [21–24,29] in which HDL supplied cells with lipids, but it is unclear which types of lipids were provided by HDL via its receptor. Our data show that HDL supports optimal T-blast proliferation by supplementation of fatty acids, especially the essential fatty acids oleic and linoleic, which are necessary for cell growth and are abundant in HDL. The content of other fatty acids, such as palmitic ($C_{16:0}$) and homo- γ -linolenic ($C_{20:3,n-6}$), is also elevated in HDL-preincubated cells, indicating that these cells may also take up some non-essential fatty acids from HDL to meet their needs during proliferation. Increased phosphatidylcholine in HDL-treated

cells may reflect phospholipid incorporation, as a source of fatty acids, via HDL. On the other hand, the increased content of linoleic acid in resting T cells preincubated with HDL indicates that HDL metabolism and fatty acid consumption also occur in resting cells.

An interesting model to assess an HDL-derived fatty acid delivery was recently reported [30]. By using human erythrocytes infected by malarial parasites unable to synthesize fatty acids *de novo* from acetate during this intraerythrocytic cycle, it was observed that HDL can support parasite growth to complete cycles within the erythrocytes in serum-free medium, whereas LDL allows only for limited development (< 30%), and very-low-density lipoproteins (VLDL) are unable to support parasite growth. Thus HDL appears to be a more efficient fatty acid donor compared with LDL and VLDL, although the mechanism of HDL interaction with the cell membrane, i.e. HDL receptor involvement, was not defined [30].

The question arises as to whether the predicted HDL receptor is involved in the process of fatty acid provision by HDL. From the data reported here, the answer is positive. First, the linear increase in cell proliferation correlates with the presence of HDL in the concentration range of HDL receptor saturation (0–50 $\mu\text{g/ml}$). Second, the increase in linoleic acid in T-blasts preincubated with LDL may be the reason for the observed down-regulation of HDL receptor activity on T-blasts, although cellular cholesterol content is higher. It is impossible to explain HDL receptor regulation simply on the basis of cellular cholesterol levels in lymphocytes. Third, in serum-free medium, factors promoting T-cell growth, such as PHA or macrophage-conditioned medium, induce up-regulation of HDL receptor activity [10], suggesting that T-blasts need more fatty acids and may meet this demand by increased HDL receptor expression. Finally, when the cells are directly 'loaded' with oleic acid or linoleic acid or phosphatidylcholine (apoA1 liposomes), HDL receptor activity is down-regulated by 30–50%, whereas exogenous stearic acid, i.e. a fatty acid that can be synthesized by the cells themselves, does not significantly affect HDL receptor expression. The mechanism of T lymphocytes 'loaded' by non-esterified fatty acids involves endocytosis of BSA–fatty acid complex and/or fatty acid/membrane lipid exchange [31,32] in the presence of high concentrations of fatty acids in culture. LDL receptor activity cannot be regulated by essential fatty acids or stearic acid, indicating that LDL uptake primarily correlates with cellular cholesterol content, whereas HDL (via its receptor) may provide essential fatty acids to lymphocytes for optimal proliferation. Increased cellular fatty acids, in turn, induce down-regulation of the HDL receptor.

Finally, although both fatty acid–albumin complexes and lipoproteins can enhance *in vitro* lymphocyte responses to mitogen stimulation, lipoproteins appear to be more effective than the complexes [9,14,31,33]. In the present experiments, a concentration of 50 μg of HDL/ml, i.e. 0.5–1 μM fatty acids, can support optimal lymphocyte proliferation induced by PHA in serum-free medium, whereas 5000 μg of BSA/ml, corresponding to approx. 25 μM fatty acids, does so, as reported by Cuthbert & Lipsky [14]. Two pathways, HDL-receptor-dependent (specific) and -independent (non-specific), of fatty acid incorporation into T-cells may be involved *in vivo*, since a fraction of fatty acids in a non-esterified form bound to albumin is present in the plasma. Interstitial fluid and lymph concentrations of lipoproteins have been estimated to range between 10 and 30% of the corresponding serum values [34,35], whereas the concentrations of HDL used in our experiments are < 10% of normal serum concentrations. Therefore, physiologically relevant concentrations of HDL support optimal T-cell proliferation compared with fatty acid/albumin complexes with ratios exceeding the

maximal physiological level of 2:1 [36]. Although it is difficult to relate observations *in vitro* directly to conditions *in vivo*, the present data support the conclusion that HDL are a more effective source of exogenous fatty acids necessary for lymphocyte growth than is albumin.

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