Naturally occurring and bioengineered apoA-I mutations that inhibit the conversion of discoidal to spherical HDL: the abnormal HDL phenotypes can be corrected by treatment with LCAT

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In the present study we have used adenovirus-mediated gene transfer of apoA-I (apolipoprotein A-I) mutants in apoA-I^{-/-} mice to investigate how structural mutations in apoA-I affect the biogenesis and the plasma levels of HDL (high-density lipoprotein). The natural mutants apoA-I(R151C)_{Paris}, apoA-I(R160L)_{Oslo} and the bioengineered mutant apoA-I(R149A) were secreted efficiently from cells in culture. Their capacity to activate LCAT (lecithin:cholesterol acyltransferase) *in vitro* was greatly reduced, and their ability to promote ABCA1 (ATP-binding cassette transporter A1)-mediated cholesterol efflux was similar to that of WT (wild-type) apoA-I. Gene transfer of the three mutants in apoA-I^{-/-} mice generated aberrant HDL phenotypes. The total plasma cholesterol of mice expressing the apoA-I(R160L)_{Oslo}, apoA-I(R149A) and apoA-I(R151C)_{Paris} mutants was reduced by 78, 59 and 61 % and the apoA-I levels were reduced by 68, 64 and 55 % respectively, as compared with mice expressing the WT apoA-I. The CE (cholesteryl ester)/TC (total cholesterol) ratio of HDL was decreased and the apoA-I was distributed in the HDL3 region.

apoA-I(R160L)_{Oslo} and apoA-I(R149A) promoted the formation of preβ1 and α4-HDL subpopulations and gave a mixture of discoidal and spherical particles. apoA-I(R151C)_{Paris} generated subpopulations of different sizes that migrate between preβ and α-HDL and formed mostly spherical and a few discoidal particles. Simultaneous treatment of mice with adenovirus expressing any of the three mutants and human LCAT normalized plasma apoA-I, HDL cholesterol levels and the CE/TC ratio. It also led to the formation of spherical HDL particles consisting mostly of α-HDL subpopulations of larger size. The correction of the aberrant HDL phenotypes by treatment with LCAT suggests a potential therapeutic intervention for HDL abnormalities that result from specific mutations in apoA-I.

Key words: adenovirus, apolipoprotein A-I (apoA-I), ATP-binding cassette transporter A1 (ABCA1), gene transfer, high-density lipoprotein (HDL) biogenesis, lecithin:cholesterol acyltransferase (LCAT).

INTRODUCTION

apoA-I (apolipoprotein A-I) is the major protein component of HDL (high-density lipoprotein) and plays an essential role in the biogenesis, structure, function and plasma concentration of HDL [1–6]. HDL is synthesized through a complex pathway that involves various membrane-bound proteins, plasma enzymes and lipid transfer proteins [7].

Several naturally occurring apoA-I mutations that produce pathological phenotypes have been described [7–12]. It has been estimated that structural mutations of apoA-I occur in 0.3 % of the Japanese population and may affect the plasma HDL levels. Seventeen out of a total of 46 naturally occurring apoA-I mutants have reduced capacity to activate LCAT (lecithin:cholesterol acyltransferase). These mutations are clustered predominantly in or at the vicinity of helix 4 of apoA-I, based on the new X-ray crystal structure [13]. Some of them predispose to atherosclerosis [8–12,14]. In recent studies, using adenovirus-mediated gene transfer in apoA-I^{-/-} mice, we were able to identify mutations in apoA-I that affected different steps in the biogenesis of HDL. This includes mutations that affect the apoA-I–ABCA1 (ATP-binding cassette transporter A1) interactions and prevent formation of α-HDL particles [15], mutations that affect the activation of LCAT, which is required for the esterification of the cholesterol of the HDL particles [16], mutations that affect the activity of the plasma

PLTP (phospholipid transfer protein) and cause dyslipidaemia [17] and mutations that cause hypertriglyceridaemia [17,18].

The objective of the present study was to investigate how important mutations in helix 4 of apoA-I [13] affect the plasma HDL levels. We have focused on two naturally occurring apoA-I mutations [apoA-I(R151C)_{Paris} and apoA-I(R160L)_{Oslo}] and one bioengineered mutation apoA-I(R149A). Replacement of Arg¹⁴⁹ by non-charged residues diminishes the only significant positive electrostatic potential that is found in the 149–160 region of apoA-I and reduces the LCAT activation capacity of the mutant protein *in vitro* [19].

Using adenovirus-mediated gene transfer of these mutants in apoA-I^{-/-} mice, we found that a common feature of the three mutations is the low HDL levels and the presence of discoidal particles in plasma. These defects could be corrected by co-infection of apoA-I^{-/-} mice with adenoviruses expressing the mutant proteins along with human LCAT, indicating that the endogenous LCAT is rate limiting in the conversion of discoidal into spherical HDL.

EXPERIMENTAL

Materials

Materials not mentioned in the Experimental section have been obtained from sources described previously [2,18].

Abbreviations used: ABCA1, ATP-binding cassette transporter A1; apoA-I, apolipoprotein A-I; CE, cholesteryl ester; cpt-cAMP, 8-(4-chlorophenylthio)-cAMP; EM, electron microscopy; GFP, green fluorescent protein; HDL, high-density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL, low-density lipoprotein; MOI, multiplicity of infection; pfu, plaque-forming units; rHDL, reconstituted HDL; TC, total cholesterol; WT, wild-type.

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Generation of adenoviruses expressing the WT (wild-type) and the mutant apoA-I forms

The apoA-I gene lacking the BglIII restriction site (that is present at nucleotide positions 181 of the genomic sequence relative to the ATG codon of the gene) was cloned into the pcDNA3.1 vector to generate the pcDNA3.1-apoA-I(Δ BglIII) plasmid (as described in Supplementary Table 1 at <http://www.BiochemJ.org/bj/406/bj4060167add.htm>). This plasmid was used as a template to introduce the apoA-I mutations apoA-I(R160L)_{Oslø}, apoA-I(R149A) and apoA-I(R151C)_{Paris} by using the QuikChange[®] XL mutagenesis kit (Stratagene). The 2.2 kb apoA-I inserts containing the WT or mutant forms of apoA-I were cloned into the pAdTrackCMV vector, which was used to generate the adenoviral constructs by recombination in the bacteria cells BJ-5183-AD1, following the pAd-Easy-1 protocol (Stratagene). Correct clones were propagated in DH5a bacteria cells. The recombinant adenoviral constructs were linearized after digestion with PacI and used to transfect 911 cells. Following large-scale infection of HEK-293 (human embryonic kidney 293) cell cultures, the recombinant adenoviruses were purified by two consecutive CsCl ultracentrifugation steps, dialysed and titrated. The first centrifugation was carried out at 35 000 rev./min for 90 min using an SW41 Beckman rotor and the second at 50 000 rev./min for 16 h using a Ti70 Beckman rotor.

Cell secretion of WT and mutant apoA-I forms

To assess the secretion of WT and mutant apoA-I forms, HTB13 cells (SW 1783 human astrocytoma) grown to 80 % confluence in Leibovitz's L-15 medium containing 2 % (v/v) heat-inactivated horse serum in 100 mm diameter dishes were infected with adenoviruses expressing the WT or mutant apoA-I form at an MOI (multiplicity of infection) of 20. Twenty-four hours post-infection, the cells were washed twice with PBS and incubated in serum-free medium for 2 h. Following an additional wash with PBS, fresh serum-free medium was added. After 24 h of incubation, medium was collected and analysed by SDS/PAGE for apoA-I expression.

Production and purification of WT and mutant apoA-I forms using the adenovirus system

apoA-I was purified from the culture medium of HTB13 cells grown in roller bottles. The cells were infected with the adenoviruses expressing the WT apoA-I, apoA-I(R149A), apoA-I(R160L)_{Oslø} or apoA-I(R151C)_{Paris}. The medium was harvested every 24 h after infection and the protein was purified as described in [4].

LCAT activation assay

LCAT was purified as described in [18] from the culture medium of human HTB13 cells infected with an adenovirus expressing the human LCAT cDNA [20]. The rHDL (reconstituted HDL) particles, used as the substrate, contained cholesterol and [¹⁴C]-cholesterol ([4-¹⁴C]cholesterol; 0.04 mCi/ml; specific activity of 45 mCi/mmol; PerkinElmer Life Sciences), POPC (1-palmitoyl-2-oleoyl phosphatidylcholine) and apoA-I and were prepared by the sodium cholate dialysis method as described previously [21]. The enzymatic reactions were carried out as described and the apparent $V_{\max}$ and K_m were derived as described previously [16].

ABCA1-dependent cholesterol efflux assay

ABCA1-dependent efflux of cholesterol was measured by using J774 macrophages in which expression of ABCA1 was induced

by a cAMP analogue using WT and mutant apoA-I forms as cholesterol acceptors. The J774 mouse macrophages were grown in DMEM (Dulbecco's modified Eagle's medium) containing 4.5 g/l glucose, 10 % (v/v) foetal bovine serum and antibiotics, labelled with 8 μ Ci/ml [¹⁴C]cholesterol for 24 h and then treated with 0.3 mM cpt-cAMP [8-(4-chlorophenylthio)-cAMP] for 24 h. Cholesterol efflux was determined as described previously [2].

Animal studies, plasma lipids and apoA-I

apoA-I^{-/-} (apoA-I^{tm1Unc}) C57BL/6J mice [22] were purchased from Jackson ImmunoResearch Laboratories (Bar Harbor, ME, U.S.A.). The mice were maintained on a 12 h light/12 h dark cycle and standard rodent chow. All procedures performed on the mice were in accordance with NIH (National Institutes of Health) and institutional guidelines. apoA-I^{-/-} mice 6–8 weeks of age were injected via tail vein with $1-2 \times 10^9$ pfu (plaque-forming units) of recombinant adenovirus per animal and the animals were killed 4 days post-injection following a 4 h fast. The concentration of TC (total cholesterol), free cholesterol, phospholipids and triacylglycerols of plasma drawn 4 days post-infection was determined by using the cholesterol E, free cholesterol C and phospholipids B reagents (Wako Chemicals) and INFINITY triacylglycerols reagent (ThermoDMA) respectively according to the manufacturer's instructions. The concentration of CE (cholesteryl esters) was determined by subtracting the concentration of free cholesterol from the concentration of TC. Plasma apoA-I levels were determined using the AutoKit-AI (Wako Chemicals). For FPLC analysis of plasma, 17 μ l of plasma obtained from mice infected with adenovirus-expressing WT or mutant apoA-I forms was loaded on to a Sepharose 6 PC column in a SMART microFPLC system (Amersham Biosciences) and eluted with PBS. A total of 25 fractions of 50 μ l volume each were collected for further analysis. The concentration of lipids in the FPLC fractions was determined as described above.

Fractionation of plasma by density-gradient ultracentrifugation and EM (electron microscopy) analysis of the apoA-I-containing fractions

For this analysis, 300 μ l of plasma obtained from adenovirus-infected mice was fractionated by density-gradient ultracentrifugation as described in [16]. Aliquots of 100 μ l from each fraction were subjected to SDS/PAGE and the protein bands were visualized by staining with Coomassie Brilliant Blue. For EM analysis, the fractions that float in the HDL region were treated and photographed as described in [16].

Non-denaturing two-dimensional gel electrophoresis

The distribution of HDL subfractions in plasma was analysed by two-dimensional electrophoresis as described in [16]. The proteins were transferred to a nitrocellulose membrane and apoA-I was detected using the goat polyclonal anti-human apoA-I antibody AB740 (Chemicon International).

RNA isolation and hybridization analysis

Total RNA was isolated from liver tissue of mice 4 days post-infection, using TRIzol[®] (Invitrogen) according to the manufacturer's instructions. The RNA was analysed by Northern blotting and the apoA-I and GAPDH (glyceraldehyde-3-phosphate

dehydrogenase) mRNA bands were detected and quantified as described in [18].

RESULTS

Protein expression and ability of mutant apoA-I forms to activate LCAT and promote ABCA1-mediated cholesterol efflux *in vitro*

We have generated recombinant adenoviruses expressing the WT apoA-I and apoA-I(R151C)_{Paris}, apoA-I(R160L)_{Oslo} and apoA-I(R149A) mutants and studied the properties of the mutant proteins *in vitro* and *in vivo*. The first two mutations are encountered in human patients [23,24]. The third represents a newly bio-engineered variant. To assess the expression and secretion of the three mutants, compared with WT apoA-I, we infected HTB13 (human astrocytoma) cells with recombinant adenoviruses harbouring the WT or the mutant apoA-I genes, using an MOI of 20. Analysis of the cultured media 24 h post-infection showed that the WT and the mutant forms were secreted at comparable levels in the medium (Figure 1A).

For the LCAT activation, WT and the three mutant forms of apoA-I were produced by the adenovirus expression system and were used for generation of discoidal rHDL particles, as described in [17,18]. The LCAT activity was assayed as the rate of production of labelled CEs from the [¹⁴C]cholesterol-labelled rHDL particles. The LCAT-catalysed esterification of the cholesterol of the rHDL particles containing the apoA-I(R151C)_{Paris} was performed with normal concentration of LCAT in the reaction mixture. The initial velocity of the LCAT-catalysed esterification of the cholesterol of the rHDL particles containing the apoA-I(R160L)_{Oslo} and apoA-I(R149A) mutants when performed with normal LCAT concentration was very low, but could be increased proportionately by increasing the concentration of LCAT in the reaction mixture. We found that a 20-fold increase in enzyme concentration using the apoA-I(R160L)_{Oslo} and apoA-I(R149A) mutants resulted in an initial velocity of esterification that was comparable with that obtained using 1-fold enzyme concentration and WT apoA-I. Taking into account the enzyme concentrations used, the $V_{\max}$ (app) and as well as the K_m (app) values of the mutants were calculated and compared with those of the WT apoA-I and are shown in Figure 1(B). The relative $V_{\max}/K_m$ for apoA-I(R151C)_{Paris}, apoA-I(R160L)_{Oslo} and apoA-I(R149A) were 28.4, 2.7 and 5.2 % nmol of CE · h⁻¹ · (μM of apoA-I)⁻¹ respectively (Figure 1B). Compared with WT apoA-I, mutant apoA-I(R160L)_{Oslo} and apoA-I(R149A) had greatly reduced $V_{\max}$ (app) values and 3.3- and 1.7-fold increase in K_m (app) values respectively, whereas mutant apoA-I(R151C)_{Paris} had near normal $V_{\max}$ (app) and 2.8-fold increase in K_m (app) (Figure 1B).

The ability of the three apoA-I mutants to promote ABCA1-mediated cholesterol efflux was determined by using J774 mouse macrophages that were stimulated by the cAMP analogue cpt-cAMP. The net cpt-cAMP-dependent cholesterol efflux from J774 macrophages by using the mutant proteins as cholesterol acceptors was obtained by subtracting the efflux values of the untreated cells from the efflux values of the CIPhS-cAMP-treated cells, after a 4 h incubation. The efflux values obtained for WT apoA-I were set to 100 %. This analysis showed that the cpt-cAMP-dependent (ABCA1-mediated) cholesterol efflux in the presence of apoA-I(R151C)_{Paris}, apoA-I(R160L)_{Oslo} and apoA-I(R149A) acceptor was 94, 105 and 98 % of the WT control (Figure 1C). Previous *in vitro* studies also indicated reduced LCAT activation and normal cholesterol efflux capacity for these mutants [25,26]. The *in vivo* effects of these mutations on the biogenesis of HDL were not studied previously.

Figure 1 apoA-I expression and *in vitro* functional assays

(A) SDS/PAGE analysis of 100 μl of culture medium of HTB13 cells infected with control adenovirus expressing GFP, adenovirus expressing WT or the mutants apoA-I(R151C)_{Paris}, apoA-I(R160L)_{Oslo} and apoA-I(R149A). apoA-I levels can be assessed qualitatively by comparison with the intensity of the band of a sample containing 1 μg of BSA. 'M' indicates protein markers. (B) Activation of LCAT by rHDL containing WT or mutant apoA-I forms. The apparent K_m and $V_{\max}$ values are given at the bottom of the Figure. The catalytic efficiency of the enzyme, expressed as the $V_{\max}$ (app)/ K_m (app) ratio, is plotted and expressed as percentage of WT control. Values are the means ± S.D. for three independent experiments performed in duplicate. (C) cAMP-dependent (ABCA1-mediated) cholesterol efflux from J774 murine macrophages in the presence of WT apoA-I, apoA-I(R151C)_{Paris}, apoA-I(R160L)_{Oslo} or apoA-I(R149A), as indicated, was determined as described in the Experimental section. Values are the means ± S.D. for three independent experiments performed in duplicate.

Plasma lipid, apoA-I and hepatic apoA-I mRNA levels following adenovirus infection

Mice were infected with doses ranging from 1 to 2×10^9 pfu in order to achieve hepatic mRNA levels that were comparable with the levels obtained in mice infected with WT apoA-I. Higher doses of the mutant forms were also used to look for potential changes in the observed phenotypes (Supplementary Table 2

Table 1 Comparison of plasma lipids and apoA-I levels and hepatic mRNA levels of apoA-I^{-/-} mice, 4 days post-infection with recombinant adenoviruses expressing the WT apoA-I, the apoA-I mutants or the control protein GFPValues are means $\pm$ S.D. ($n = 4-6$).

Protein expressed	Cholesterol (TC) (mg/dl)	Free cholesterol (mg/dl)	CEs (mg/dl)	CE/TC	Triacylglycerols	Phospholipids	Relative apoA-I mRNA (%)	apoA-I protein (mg/dl)
WT apoA-I	190 $\pm$ 43	51 $\pm$ 15	139 $\pm$ 28	0.73 $\pm$ 0.06	76 $\pm$ 16	381 $\pm$ 85	100 $\pm$ 15	204 $\pm$ 47
GFP	40 $\pm$ 5	19 $\pm$ 6	21 $\pm$ 3	0.53 $\pm$ 0.01	45 $\pm$ 4	78 $\pm$ 8	—	—
apoA-I(R151C) _{Paris}	75 $\pm$ 6	32 $\pm$ 8	42 $\pm$ 9	0.52 $\pm$ 0.08	10 $\pm$ 4	119 $\pm$ 12	63 $\pm$ 10	91 $\pm$ 11
apoA-I(R151C) _{Paris} + LCAT	437 $\pm$ 89	143 $\pm$ 22	294 $\pm$ 66	0.67 $\pm$ 0.01	104 $\pm$ 29	561 $\pm$ 86	64 $\pm$ 5	199 $\pm$ 52
apoA-I(R149A)	78 $\pm$ 10	43 $\pm$ 12	35 $\pm$ 2	0.44 $\pm$ 0.06	47 $\pm$ 8	138 $\pm$ 21	115 $\pm$ 12	75 $\pm$ 26
apoA-I(R149A) + LCAT	216 $\pm$ 67	64 $\pm$ 21	152 $\pm$ 43	0.70 $\pm$ 0.02	54 $\pm$ 6	213 $\pm$ 80	50 $\pm$ 6	188 $\pm$ 68
apoA-I(R160L) _{Oslo}	43 $\pm$ 13	33 $\pm$ 8	10 $\pm$ 3	0.23 $\pm$ 0.01	36 $\pm$ 4	116 $\pm$ 48	117 $\pm$ 30	66 $\pm$ 31
apoA-I(R160L) _{Oslo} + LCAT	250 $\pm$ 47	45 $\pm$ 7	205 $\pm$ 41	0.82 $\pm$ 0.01	62 $\pm$ 11	277 $\pm$ 73	60 $\pm$ 1	127 $\pm$ 26
Untreated mice								
apoA-I ^{-/-}	30 $\pm$ 9	11 $\pm$ 2	19 $\pm$ 6	0.63 $\pm$ 0.02	30 $\pm$ 5	52 $\pm$ 12	—	—
C57BL/6	88 $\pm$ 10	24 $\pm$ 2	64 $\pm$ 15	0.72 $\pm$ 0.05	31 $\pm$ 7	150 $\pm$ 15	—	—

of the Supplementary data at <http://www.BiochemJ.org/bj/406/bj4060167add.htm>). Treatment of apoA-I^{-/-} mice with a control adenovirus expressing the GFP (green fluorescent protein) did not change their plasma lipid levels, compared with the untreated apoA-I^{-/-} mice. Potential liver damage following adenovirus infection was assessed by measuring serum transaminases using Reflotron Plus system (Roche). These analyses showed normal serum transaminase levels when mice were infected with 2×10^9 pfu recombinant adenoviruses. Analysis of plasma lipids and apoA-I levels, 4 days post-infection, showed that, compared with mice expressing WT apoA-I, the mice expressing any of the three mutants had in general reduced total plasma cholesterol levels and reduced plasma CE/TC ratio. The triacylglycerol and phospholipid plasma levels in mice expressing the three mutants were also reduced as compared with mice expressing the WT apoA-I (Table 1).

There were differences in the lipid phenotypes of the mice expressing the three apoA-I mutants. Mice expressing the mutants apoA-I(R160L)_{Oslo}, apoA-I(R149A) and apoA-I(R151C)_{Paris} had 22, 41 and 39 % plasma cholesterol and 32, 36 and 45 % apoA-I levels respectively, as compared with mice expressing the WT apoA-I. The CE/TC ratio of the plasma of mice expressing the apoA-I(R160L)_{Oslo}, apoA-I(R149A) and apoA-I(R151C)_{Paris} mutants was 0.23, 0.44 and 0.52 respectively, indicating some kind of defect in the esterification of the HDL cholesterol due to the reduced ability of these apoA-I mutants to activate the endogenous LCAT *in vivo*.

FPLC analysis of plasma showed that the HDL cholesterol peak of the mice expressing any of the three mutants was very small compared with mice expressing the WT apoA-I, while the apoA-I mRNA levels of mice expressing these mutants and the WT apoA-I were comparable thus indicating defective biogenesis of HDL (Figure 2 and Table 1). In mice expressing the apoA-I(R151C)_{Paris} mutant the HDL cholesterol peak was shifted towards the HDL3 region (Figure 2). A similar shift was observed when higher doses of adenoviruses expressing the three mutants were used (Supplementary Figure 1 of the Supplementary data at <http://www.BiochemJ.org/bj/406/bj4060167add.htm>).

Analysis of the distribution of apoA-I following density-gradient ultracentrifugation of plasma showed that in mice expressing the WT apoA-I, the protein was equally distributed in the HDL2 and HDL3 region (Figure 3A). Consistent with the FPLC data (Figure 2), in mice expressing apoA-I(R160L)_{Oslo} and apoA-I(R149A) the apoA-I levels were reduced and most of the apoA-I protein was distributed in the HDL3 region and small

Figure 2 FPLC profiles of TC in plasma of apoA-I^{-/-} mice infected with 1×10^9 pfu of adenoviruses expressing the WT apoA-I, apoA-I(R151C)_{Paris}, apoA-I(R160L)_{Oslo}, apoA-I(R149A) or the control protein GFP

Plasma samples were obtained from mice infected with the recombinant adenoviruses, 4 days post-infection.

amounts in the HDL2 region (Figures 3B and 3C respectively). In mice expressing the apoA-I(R151C)_{Paris} mutant, the apoA-I levels were reduced to a lesser extent and the mutant apoA-I was distributed predominantly (80 %) in the HDL3 region and the remainder in the HDL2 region. Small amounts of apoA-I were also present in the lower density fractions (Figure 3D).

Analysis by EM of the HDL fraction 6, obtained by density-gradient ultracentrifugation (Figures 3A–3D), showed that WT apoA-I promoted the formation of spherical particles (Figure 3E), whereas the apoA-I(R160L)_{Oslo} and apoA-I(R149A) mutants promoted the formation of a mixture of discoidal and spherical HDL particles (Figures 3F and 3G respectively). The apoA-I(R151C)_{Paris} mutant promoted the formation of mostly spherical and fewer discoidal particles (Figure 3H). The HDL fraction obtained from mice infected with the control adenovirus expressing GFP contained only few spherical particles (Figure 3I) that were similar in numbers to the particles observed in the HDL fraction of the untreated apoA-I^{-/-} mice (results not shown).

Two-dimensional gel electrophoresis of plasma from mice expressing the WT or the mutant apoA-I forms showed that the WT apoA-I formed α -HDL particles and smaller amounts

Figure 3 Analyses of plasma of apoA-I^{-/-} mice infected with adenoviruses expressing the WT apoA-I (A, E, J), the apoA-I(R160L)_{Oslo} (B, F, K), apoA-I(R149A) (C, G, L), the apoA-I(R151C)_{Paris} (D, H, M) or the control adenovirus expressing GFP (I) by density-gradient ultracentrifugation, SDS/PAGE and two-dimensional gel electrophoresis

(A–C) SDS/PAGE analysis of density-gradient ultracentrifugation fractions. The densities of the fractions are indicated on the top of the Figure. (E–I) EM pictures of HDL fraction 6 obtained from apoA-I^{-/-} mice expressing the WT apoA-I, the mutant forms or the control adenovirus expressing GFP, following density-gradient ultracentrifugation of plasma. The arrows indicate discoidal HDL particles. (J–M) Two-dimensional (2D) gel electrophoresis and Western-blot analysis of plasma obtained from mice infected with adenoviruses expressing the WT or the mutant forms of apoA-I, as indicated. The arrows indicate the different HDL subpopulations.

of pre β ₁-HDL particles (Figure 3J). Mice expressing the apoA-I(R160L)_{Oslo} formed predominantly α 4-HDL of fast electrophoretic mobility and small amounts of pre β ₁-HDL and α 3- and α 2-HDL subpopulations with slower electrophoretic mobility (Figure 3K). The apoA-I(R149A) formed pre β ₁ and α 4-HDL particles and small amounts of α 1-, α 2- and α 3-HDL particles of slower electrophoretic mobility (Figure 3L). The apoA-I(R151C)_{Paris} formed subpopulations of different sizes that migrated between pre β and α -HDL (Figure 3M; see Supplementary Figures 2A–2D of the Supplementary data at <http://www.BiochemJ.org/bj/406/bj4060167add.htm>).

Plasma lipids, FPLC profiles, the distribution of HDL in different densities and the size and shape of HDL in mice infected with adenoviruses expressing the three mutant apoA-I forms in combination with human LCAT

To assess how apoA-I mutations affect the biogenesis of HDL, apoA-I^{-/-} mice were co-infected with a mixture of adenoviruses expressing apoA-I(R160L)_{Oslo}, apoA-I(R149A) or apoA-I(R151C)_{Paris} (using 1×10^9 pfu) along with human LCAT (using 5×10^8 pfu). The co-infection with LCAT and each of the three mutants had a dramatic effect on plasma cholesterol and apoA-I levels. Compared with mice infected with adenoviruses

expressing the mutant apoA-I forms alone, mice co-infected with adenoviruses expressing LCAT and the apoA-I(R160L)_{Oslo}, apoA-I(R149A) and apoA-I(R151C)_{Paris} mutants had a 1.9-, 2.5- and 2.2-fold increase in plasma apoA-I respectively. The increase in TC was 5.8-, 2.8- and 5.8-fold respectively, and the increase in esterified cholesterol was 21-, 4.3- and 7-fold respectively (Table 1). The LCAT treatment also normalized the CE/TC ratio (Table 1). These increases were observed despite the lower levels of hepatic apoA-I mRNA expression in mice treated with combination of LCAT and the mutant apoA-I forms, as compared with mice treated with the mutant apoA-I forms alone (Table 1).

Figures 4(A)–4(C) show the FPLC profiles of cholesterol in mice treated with a combination of human LCAT and apoA-I(R160L)_{Oslo} (Figure 4A), apoA-I(R149A) (Figure 4B) and apoA-I(R151C)_{Paris} (Figure 4C) and compare them with the profiles of mice treated with the WT or the corresponding mutant apoA-I forms alone. These analyses showed that all the increase in cholesterol could be attributed to the increase in cholesterol that floats in the HDL2 and HDL1 region [that overlaps with the LDL (low-density lipoprotein) density range]. The apoA-I(R151C)_{Paris} gave a continuous HDL peak that is shifted to the lower densities, whereas the HDL of apoA-I(R149A) and apoA-I(R160L)_{Oslo} gave two distinct peaks corresponding to HDL2 and HDL1 (Figures 4A–4C).

Figure 4 Analyses of plasma of apoA-I^{-/-} mice infected with a combination of adenoviruses expressing human LCAT (5×10^8 pfu) and apoA-I(R160L)_{Oslo} (1×10^9 pfu), apoA-I(R149A) (1×10^9 pfu) or apoA-I(R151C)_{Paris} (1×10^9 pfu) mutants

(A–C) FPLC profiles of TC and comparison with the corresponding profiles of plasma obtained from apoA-I^{-/-} mice expressing the WT apoA-I or each of the three apoA-I mutants alone, as indicated. The CE/TC ratio of HDL fractions 15–22 of the FPLC is indicated. (D–F) SDS/PAGE profiles of fractions obtained by density-gradient ultracentrifugation analysis of plasma of apoA-I^{-/-} mice expressing the mutant forms of apoA-I in combination with the human LCAT, as indicated. (G–I) EM pictures of HDL fraction 6, following density-gradient ultracentrifugation of plasma obtained from apoA-I^{-/-} mice expressing the indicated mutant forms in combination with human LCAT. (J–L) Two-dimensional gel electrophoresis and Western blot analysis of plasma obtained from mice infected with adenoviruses expressing the WT or the mutant forms of apoA-I, as indicated. The arrows indicate the different HDL subpopulations.

The LCAT treatment in mice expressing any of the three mutants also normalized the CE/TC ratio of HDL (Figures 4A–4C).

Fractionation of plasma by density-gradient ultracentrifugation and SDS/PAGE analysis of the fractions confirmed the increase in plasma apoA-I levels shown in Table 1. The plasma apoA-I for the three mutants was shifted towards the HDL2 region, whereas smaller amounts of apoA-I were found in the HDL3 region and the LDL density range (Figures 4D–4F). The distribution of apoA-I assessed by ultracentrifugation and SDS/PAGE analysis is consistent with the distribution of HDL cholesterol of the three mutants relative to WT apoA-I, as determined by FPLC analysis (compare Figures 4D–4F with Figures 4A–4C).

The co-infection of the mutant apoA-I forms with LCAT increased the concentration of apoE in the VLDL (very-low-density lipoprotein)/IDL (intermediate-density lipoprotein)/LDL region. It also increased the concentration of apoA-IV in the HDL3 and, to a lesser extent, in the HDL2 region (compare Figures 3B and 3C with Figures 4D and 4E).

The LCAT treatment promoted the formation of spherical HDL particles similar to those formed by WT apoA-I (compare

Figure 3E with Figures 4G–4I) and restored the larger size HDL subpopulations in the plasma. In mice infected with the apoA-I(R160L)_{Oslo} and apoA-I(R149A) the pre β -HDL disappeared and the relative concentration of the α 2, α 3 and α 1 particles increased, whereas in mice expressing the apoA-I(R151C)_{Paris} the pre β to α -HDL ratio appears normal (Figures 4J–4L).

DISCUSSION

In previous studies, we considered the HDL biogenesis as a continuous pathway where apoA-I and various participating proteins interact successively to form initially pre β -HDL and subsequently discoidal and spherical HDL particles that are biologically active [7]. Gene transfer of apoA-I mutants to apoA-I^{-/-} mice identified discrete steps in the HDL pathway, where intermediates cannot be converted into products and therefore accumulate in plasma [2, 16–18, 27] or are degraded [15].

In the present study, we used the same strategy to assess the impact of two naturally occurring apoA-I mutations, apoA-I(R160L)_{Oslo} and apoA-I(R151C)_{Paris}, and a newly bioengineered

mutation, apoA-I(R149A), on the biogenesis of HDL. Both Arg¹⁶⁰ and Arg¹⁴⁹ are thought to contribute to the positive electrostatic potential of apoA-I [19]. The phenotypes that emerged following adenovirus-mediated gene transfer of the naturally occurring apoA-I mutants in apoA-I^{-/-} mice are expected to be similar to the phenotypes of homozygous subjects carrying either of the two natural apoA-I mutations [23,24]. Currently, only the phenotypes of heterozygotes patients are available. In addition, animal models that express transiently the mutant proteins may be used to identify potential therapies for the low HDL syndromes. In our studies, the formation of HDL was assessed by FPLC fractionation and two-dimensional gel electrophoresis of plasma that can identify potential defects in the formation of HDL and imbalances in the pre β and α -HDL subpopulations [17]. Other parameters that indicate abnormalities in HDL biogenesis are the CE/TC ratio of HDL, the skewed distribution of apoA-I towards the HDL3 region and the ability of the mutant proteins to drive the formation of spherical and/or discoidal HDL particles as determined by EM [16,17]. The function of the apoA-I mutants was also assessed by two informative *in vitro* assays.

The capacity of apoA-I mutants to promote ABCA1-mediated cholesterol efflux indicates potential defects in the functional interactions between apoA-I and ABCA1 that lead to the initial lipidation of apoA-I [28]. The capacity of apoA-I to esterify the cholesterol of rHDL particles may indicate potential defects that inhibit the maturation of the lipidated apoA-I and the formation of spherical HDL particles. Consistent with previous findings [25,26,29,30], our *in vitro* assays showed that the three mutants had near normal capacity to promote ABCA1-mediated lipid efflux and greatly reduced capacity to activate LCAT.

Gene transfer of apoA-I(R160L)_{Oslo} and apoA-I(R149A) mutants in apoA-I^{-/-} mice resulted in a great reduction of total plasma apoA-I and HDL cholesterol levels, as compared with WT apoA-I. It also promoted formation of pre β -HDL and small size α 4-HDL particles, accumulation of discoidal HDL particles in plasma, decreased CE/TC ratio of the plasma and the HDL fraction and caused a shift of apoA-I and HDL towards the HDL3 region. The phenotypes produced by adenovirus-mediated gene transfer of apoA-I(R149A) or apoA-I(R160L)_{Oslo} were similar. A similar phenotype has been previously produced by replacement of Arg¹⁶⁰/His¹⁶² by valine and alanine respectively [16].

Our findings are consistent with previous studies that showed that heterozygotes of apoA-I(R160L)_{Oslo} have approx. 60 and 70 % of normal HDL and apoA-I levels respectively, form pre β 1 and small size α -HDL particles and have a 30 % reduction in their plasma LCAT activity [24].

Heterozygotes for apoA-I(R151C)_{Paris} had approx. 50 % of normal plasma HDL cholesterol and apoA-I levels, reduced plasma LCAT activity and a shift of HDL towards the HDL3 region [23]. Carriers of apoA-I(R151C)_{Paris} mutation did not have manifestations of coronary heart disease [23]. This may be the result of the antioxidant properties of this mutant that has a free cysteine moiety located near the interface of the hydrophobic/hydrophilic surface of the α -helix [31].

LCAT treatment can correct the HDL abnormalities brought about by the mutations apoA-I(R160L)_{Oslo}, apoA-I(R151C)_{Paris} and apoA-I(R149A) and suggest a mechanism responsible for the HDL deficiency observed in human patients carrying the two natural apoA-I mutations

Insights into the molecular aetiology of the low HDL levels observed in patients with apoA-I(R160L)_{Oslo} and apoA-I(R151C)_{Paris} were provided by treatment of apoA-I^{-/-} mice with a combin-

ation of adenoviruses expressing apoA-I(R160L)_{Oslo}, apoA-I(R151C)_{Paris} or apoA-I(R149A) and the human LCAT. This treatment corrected the plasma apoA-I and HDL levels, normalized the CE/TC ratio, restored normal pre β - and α -HDL subpopulations and led to the formation of spherical HDL particles.

The restoration of normal levels of apoA-I and HDL following treatment with LCAT supports the notion that apoA-I mutants are secreted efficiently by the liver but the ability of the endogenous LCAT to esterify the cholesterol of the discoidal particles is rate limiting. Due to the LCAT insufficiency, the discoidal HDL accumulates in plasma. The reduction of the plasma apoA-I and HDL levels most likely represents increased catabolism of the discoidal particles via the kidney by the cubilin receptor or other mechanisms [32,33].

In the presence of excess LCAT, the esterification of the cholesterol of the discoidal HDL particles promotes their conversion into spherical HDL, thus reducing the overall catabolism of HDL.

The diminished capacity of apoA-I mutants carrying amino acid substitutions in the 149–160 region to activate LCAT has been supported by the *in vitro* data of this and several previous studies [19,24–26,29,34]. It has been suggested that Arg¹⁶⁰ plays a direct role in the activation of LCAT [29], since mutagenesis of this residue does not change the structure and thermodynamic properties of the mutant protein, but has a major effect on the activation of LCAT [19,29]. Similarly, an Arg¹⁴⁹ to glutamic residue substitution had no effect on the structure and the thermodynamic stability of apoA-I but diminished the capacity of the mutant protein to activate LCAT [19].

It has been suggested that Arg¹⁴⁹, Arg¹⁵³ and Arg¹⁶⁰, which are located in the hydrophobic/hydrophilic boundary of the amphipathic helix, create the only significant positive electrostatic potential around apoA-I [19]. In the ‘belt’ model these residues do not form interhelical salt bridges with the corresponding antiparallel repeat 99–120 but can form either intrahelical salt bridges or can be free to have electrostatic interactions with LCAT and thus promote its activation [19].

When Arg¹⁴⁹ or Arg¹⁶⁰ are replaced by alanine or other hydrophobic residues, the overall hydrophobicity increases and the positive electrostatic potential of this region decreases. The reduction in the positive electrostatic potential of these mutants may account for their diminished ability to activate LCAT *in vitro* and *in vivo*. The diminished capacity of apoA-I(R151C)_{Paris} to activate efficiently LCAT *in vitro* and *in vivo* may be related to a different registration of the two antiparallel apoA-I helices as compared with WT apoA-I, due to the formation of a disulfide bond between the two Cys¹⁵¹ residues [35].

Taken together with previous studies, our data suggest the following events that account for the abnormal HDL phenotypes and the low HDL levels of humans or experimental animals expressing apoA-I(R160L)_{Oslo}, apoA-I(R151C)_{Paris} and apoA-I(R149A). Following the initial lipidation of apoA-I by ABCA1, the complex proceeds to form discoidal particles. However, the activity of LCAT is rate limiting and the discoidal particles cannot be converted efficiently into spherical particles. This defective conversion is associated with low HDL levels, most likely due to the increased catabolism of the discoidal HDL particles [32,33]. The correction of the aberrant HDL phenotypes by treatment with LCAT suggests a potential therapeutic intervention for HDL abnormalities that result from specific mutations in apoA-I. Experimental interventions in animal models may involve, for instance, treatments that increase the expression of the endogenous LCAT gene and/or potential gene therapy in the future. Such treatments will have as their target not only the increase in HDL levels but also the protection from atherosclerosis.

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