Reversal of hyperlipidaemia in apolipoprotein C1 transgenic mice by adenovirus-mediated gene delivery of the low-density-lipoprotein receptor, but not by the very-low-density-lipoprotein receptor

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We have shown previously that human apolipoprotein (apo)C1 transgenic mice exhibit hyperlipidaemia, due primarily to an impaired clearance of very-low-density lipoprotein (VLDL) particles from the circulation. In the absence of at least the low-density-lipoprotein receptor (LDLR), it was shown that *APOC1* overexpression in transgenic mice inhibited the hepatic uptake of VLDL via the LDLR-related protein. In the present study, we have now examined the effect of apoC1 on the binding of lipoproteins to both the VLDL receptor (VLDLR) and the LDLR. The binding specificity of the VLDLR and LDLR for apoC1-enriched lipoprotein particles was examined *in vivo* through adenovirus-mediated gene transfer of the VLDLR and the LDLR [giving rise to adenovirus-containing (Ad)-VLDLR and Ad-LDLR respectively] in *APOC1* transgenic mice, LDLR-deficient ($LDLR^{-/-}$) mice and wild-type mice. Remarkably, Ad-VLDLR treatment did not reduce hyperlipidaemia in transgenic mice overexpressing human *APOC1*, irrespective of both the level of transgenic expression and the presence of the LDLR, whereas Ad-VLDLR treatment did reverse hyperlipidaemia in $LDLR^{-/-}$ and wild-type mice. On the other hand, Ad-LDLR treatment strongly decreased plasma lipid levels in these *APOC1* transgenic mice. These results suggest that apoC1 inhibits the clearance of lipoprotein particles via the VLDLR, but not via the LDLR. This hypothesis is corroborated by *in vitro* binding studies. Chinese hamster ovary (CHO) cells expressing the VLDLR (CHO-VLDLR) or LDLR (CHO-LDLR) bound less *APOC1* transgenic VLDL than wild-type VLDL. Intriguingly, however, enrichment with apoE enhanced dose-dependently the binding of wild-type VLDL to CHO-VLDLR cells (up to 5-fold), whereas apoE did not enhance the binding of *APOC1* transgenic VLDL to these cells. In contrast, for binding to CHO-LDLR cells, both wild-type and *APOC1* transgenic VLDL were stimulated upon enrichment with apoE. From these studies, we conclude that apoC1 specifically inhibits the apoE-mediated binding of triacylglycerol-rich lipoprotein particles to the VLDLR, whereas apoC1-enriched lipoproteins can still bind to the LDLR. The variability in specificity of these lipoprotein receptors for apoC1-containing lipoprotein particles provides further evidence for a regulatory role of apoC1 in the delivery of lipoprotein constituents to different tissues on which these receptors are located.

Key words: apolipoprotein C, gene therapy, lipoproteins, receptors.

INTRODUCTION

Apolipoprotein (apo)C1 is a small (6.6 kDa) protein component of chylomicrons, very-low-density lipoproteins (VLDLs) and high-density lipoproteins (HDLs). Previous studies *in vitro* have showed that the addition of human apoC1 to chylomicrons [1] and triacylglycerol (TG) emulsions [2] inhibits their uptake by perfused rat livers. Ligand-blotting assays showed that apoCs inhibited the apoE-mediated binding of β-VLDL to the low-density-lipoprotein receptor (LDLR)-related protein (LRP), with apoC1 being the most effective ligand [3,4]. This inhibitory action of apoC1 on lipoprotein binding to LRP was suggested to be due to displacement of apoE from the lipoprotein particle. In accordance with these studies, it was shown that synthetic peptides of apoC1 were able to displace significant amounts of apoE from β-VLDL and inhibit the binding of β-VLDL to LRP [5]. In addition, others have reported that apoC1 hampers the apoE-mediated binding of VLDL to the LDLR, either by masking apoE or by changing the conformation of apoE [6]. Thus from these studies *in vitro* it can be concluded that apoC1 inhibits the uptake of TG-rich lipoproteins by the liver, owing to an impaired binding of the respective lipoproteins to either the LDLR or LRP.

The generation of transgenic mice overexpressing the human *APOC1* gene have enabled us to study directly the influence of apoC1 on lipoprotein clearance *in vivo*. *APOC1* transgenic mice have pronounced elevated levels of cholesterol and TG, owing to an impaired hepatic uptake of VLDL [7–10]. Thus a direct inhibitory effect of apoC1 on the clearance of TG-rich lipoproteins holds true for the situation *in vivo* as well. In a combination of experiments in which *APOC1* transgenic mice were cross-bred with LDLR-knock-out mice, and transfections were performed with adenovirus containing the receptor-associated protein (*RAP*) gene, it was shown that, at least in the absence of the LDLR, apoC1 inhibited the hepatic uptake of VLDL via LRP [9].

In the present study, we have characterized the effect of apoC1 on the binding of VLDL to other members of the LDLR family,

Abbreviations used: apo, apolipoprotein; (V)LDL, (very)-low-density lipoprotein; HDL, high-density lipoprotein; TG, triacylglycerol; LDLR, LDL receptor; LRP, LDLR-related protein; RAP, receptor-associated protein; VLDLR, VLDL receptor; IDL, intermediate-density lipoprotein; LPL, lipoprotein lipase; FFA, free fatty acids; β-Gal, β-galactosidase; Ad-, adenovirus-containing; CHO, Chinese hamster ovary; PFU, plaque-forming units.

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i.e. the VLDL receptor (VLDLR) and LDLR. The VLDLR is very similar in structure to the LDLR itself, but has a different expression pattern among tissues. Whereas the LDLR is expressed abundantly in the liver, it has been reported that the VLDLR is highly expressed in heart, skeletal muscle and adipose tissue [11–13]. Immunolocalization studies showed that the VLDLR is present on the endothelium of capillaries and small arterioles [14]. The VLDLR binds with high affinity particles containing apoE, such as chylomicrons, VLDLs and intermediate-density lipoproteins (IDLs), but not LDLs [11,15]. In addition to apoE, lipoprotein lipase (LPL) was found to enhance the binding of lipoproteins to the VLDLR [15,16]. RAP, a 39 kDa protein known to inhibit ligand binding to all members of the LDLR family, was also endocytosed by the VLDLR [17] and inhibited ligand binding to it [18]. Owing to its localization and ligand specificity, it is hypothesized that the VLDLR facilitates the binding of TG-rich particles and subsequent delivery of free fatty acids (FFA) to tissues active in FFA metabolism [15,19,20]. In accordance with this hypothesis, mice deficient in the VLDLR have a 50 % lower adipose-tissue mass, as determined by the mass of epididymal fat-pads [21].

To investigate the specificity of the VLDLR and LDLR for apoC1-enriched lipoprotein particles *in vivo*, either the VLDLR or the LDLR was overexpressed in the liver of human *APOC1* transgenic mice using adenovirus-mediated gene transfer. Data presented here show that apoC1 inhibits lipoprotein binding to the VLDLR *in vivo* as well as *in vitro*, whereas apoC1-enriched lipoproteins are able to bind to the LDLR.

MATERIALS AND METHODS

Animals

Transgenic mice with high expression of human *APOC1* were generated as described previously [9]. *APOC1* transgenic mice were bred with C57BL/6J mice, from which lines 11/1 and 11/3 of the F_3 and F_4 generations respectively were used in all experiments. Female transgenic mice of line 11/3 were cross-bred with male LDLR-deficient ($LDLR^{-/-}$) mice, purchased from the Jackson laboratory (Bar Harbor, ME, U.S.A.), to obtain $11/3 \times LDLR^{-/-}$ mice. All mice in the present study were housed under standard conditions with free access to water and a standard mouse–rat (Chow) diet. Experiments were performed at 13:00 h, with food withdrawn at 9:00 h.

Plasma lipid and lipoprotein analysis

Levels of total plasma cholesterol and TG (without measuring free glycerol) were determined using commercially available enzymic kits no. 236691 (Boehringer Mannheim GmbH, Mannheim, Germany) and no. 337-B (Sigma GPO-Trinder kit, St. Louis, MO, U.S.A.).

For FPLC fractionation, 200 μl of pooled plasma per group was injected on to two 25-ml Superose 6 preparation-grade columns (connected in series; Pharmacia, Uppsala, Sweden), and eluted at a constant flow rate of 0.5 ml/min with PBS, pH 7.4. Fractions of 0.5 ml were collected and assayed for total cholesterol and TG, as described above.

Lipoprotein isolation and labelling

Either VLDL ($d < 1.006$ g/ml) or VLDL/IDL ($d < 1.019$ g/ml) fractions were isolated from pooled serum of at least eight mice per group by sequential ultracentrifugation at 40000 rev./min in a SW-40 swing-out rotor (Beckman, Geneva, Switzerland) for 18 h at 5 °C. Each lipoprotein fraction was dialysed against PBS, pH 7.4, at 4 °C overnight. Protein concentrations in the lipid fractions were determined by the method of Lowry et al. [22] with BSA as the standard.

For clearance studies *in vivo* and binding studies *in vitro*, the respective lipoprotein fractions were radiolabelled with ^{125}I by the iodine monochloride method [23]. The specific radioactivity of the labelled lipoproteins ranged from 124–253 c.p.m. per ng of protein. After iodination, the lipoprotein samples were dialysed four times against PBS, pH 7.4, and then stored at 4 °C and used within 1 week.

Enrichment of VLDL with apoE

APOC1 transgenic VLDL (line 11/1) and wild-type VLDL fractions were incubated with increasing amounts of recombinant apoE3, as indicated at 37 °C for 1 h. Thereafter, apoE-enriched VLDL was reisolated by ultracentrifugation as described above, to remove all apoE that was not associated with the VLDL fractions.

The amounts of apoE associated with VLDL were measured by 'sandwich' ELISA [7]. Briefly, affinity-purified polyclonal goat anti-(human apoE) antibodies were used for coating the plates. After incubation with the lipoprotein fractions, affinity-purified polyclonal goat anti-(human apoE) antibodies conjugated to horseradish peroxidase were used as secondary antibodies. Detection was performed by using the immunoperoxidase procedure, using tetramethylbenzidine as the substrate. Pooled plasma from healthy human subjects with a known level of apoE was used as a standard.

SDS/PAGE

VLDL ($d < 1.006$ g/ml) fractions of each group were analysed for apolipoprotein composition by SDS/PAGE by using 4–25 % gradient gels. Proteins were either stained with Serva Blue R or transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) followed by incubation with polyclonal rabbit antisera raised against both human and mouse apoE and apoC1. Donkey anti-(rabbit ^{125}I-IgG) (Amersham Corp., Braunschweig, Germany) was used as a secondary antibody, and detection was performed by scanning the blots with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Adenovirus injections

The generation of recombinant adenoviral vectors expressing human VLDLR [24], human LDLR [25] or the β-galactosidase (β-Gal) gene [26], under the control of the cytomegalovirus promoter, has been described previously. Adenovirus-containing (Ad)-LDLR and Ad-β-Gal were kindly provided by Dr. J. Herz (Department of Molecular Genetics, UT Southwestern, Dallas, TX, U.S.A.). The recombinant adenovirus was propagated and titrated in a similar way to that described previously [9]. For adenovirus administration *in vivo*, the virus was purified twice by CsCl-gradient centrifugation, followed by extensive dialysis against TD buffer [25 mM Tris/HCl/137 mM NaCl/5 mM KCl/0.73 mM Na_2HPO_4/0.9 mM $CaCl_2$/0.5 mM $MgCl_2$ (pH 7.45)] at 4 °C. After dialysis, mouse serum albumin was added to 0.2 % and glycerol to 10 % (both w/v), and the virus stocks were then frozen in aliquots in liquid N_2 and stored at −80 °C. Routine virus titres of the stocks varied from $(1–5) \times 10^{10}$/ml. On day 0, 3×10^9 plaque-forming units (PFU) in a total vol. of 200 μl (diluted with PBS) were injected into the tail vein of the mice. Similar to studies published previously [27], blood samples were drawn from the tail vein of fasting mice 5 days after virus injection.

Clearance of ^{125}I-apoB *in vivo*

ApoB removal in mice was examined essentially as described previously [28]. Fasted mice were injected with ^{125}I-labelled autologous VLDL/IDL (10 μg of tracer) in 200 μl of 0.9 % (w/v) NaCl containing 2 mg/ml BSA into their tail vein. Blood samples of approx. 50 μl were collected from the tail vein at the indicated time points after the injection. The plasma content of ^{125}I-labelled apoB was determined following propan-2-ol precipitation and measurement of the ^{125}I content of the pellet.

Binding of VLDL to Chinese hamster ovary (CHO) cells

VLDL binding was studied in CHO cells expressing the VLDLR (CHO-VLDLR), the LDLR (CHO-LDLR) [29] or lacking both receptors (CHO$^{-/-}$). CHO cells were cultured in Ham's F10 medium supplemented with 10 % (v/v) fetal-calf serum, streptomycin (200 μg/ml), penicillin (200 units/ml) and L-glutamine (2 mM). The CHO cells were incubated at 37 °C in an atmosphere containing 5 % CO_2 and grown for each uptake experiment in 12-well plates. Ham's F10 medium supplemented with 5 % (v/v) lipoprotein-deficient serum, instead of fetal-calf serum, was added to the cells 24 h before the start of the experiment.

The receptor-mediated binding of ^{125}I-labelled VLDL to CHO-LDLR or CHO-VLDLR cells, or to CHO$^{-/-}$ as a control, was determined after a 3 h incubation at 4 °C with 10 μg/ml of the respective ^{125}I-labelled lipoproteins, in either the presence or absence of a 20-fold excess of unlabelled lipoproteins. Thereafter, the cells were washed four times with 1.5 ml of PBS containing 0.1 % (w/v) BSA, followed by one wash with PBS alone. Binding at 4 °C was measured, as described previously [30].

RESULTS

Adenovirus-mediated *VLDLR* and *LDLR* gene transfer in *APOC1* transgenic mice

To study the interaction of apoC1 with the VLDLR *in vivo*, the VLDLR was ectopically expressed in the liver of *APOC1* transgenic mice by injecting mice with an adenovirus containing the *VLDLR* gene (Ad-VLDLR; 3×10^9 PFU). We reported previously [9,10] plasma cholesterol and TG levels for *APOC1* transgenic mice of lines 11/3 and 11/1. These levels correspond to a combined hyperlipidaemic phenotype in both lines of mice. Northern blot analysis demonstrated that mice of line 11/1 (high-expresser) had 1.7-fold elevated levels of human mRNA in the liver, as compared with 11/3 mice (low-expresser). In addition, we recently have reported on breeding low-expresser 11/3 mice with LDLR knock-out (LDLR$^{-/-}$) mice to obtain homozygous LDLR knock-out mice overexpressing human *APOC1* (11/3 × LDLR$^{-/-}$ mice) [9]. As a first step, the effectiveness of the virus containing the *VLDLR* gene was examined in LDLR$^{-/-}$ mice and in LDLR$^{-/-}$ mice overexpressing human *APOC1*. Since these mice obviously lack the LDLR, changes in plasma lipid levels are expected to be due to overexpression of the *VLDLR*, rather than to aberrant expression of the *LDLR* gene, which might possibly be caused by perturbations during the experiment. Ad-β-Gal injections (3×10^9 PFU) were used as a control. Similar to results reported previously [24,31], plasma cholesterol levels were decreased by 60 % in Ad-VLDLR-treated LDLR$^{-/-}$ mice, as compared with Ad-β-Gal-treated LDLR$^{-/-}$ mice (Table 1). TG levels in LDLR$^{-/-}$ mice were not affected upon treatment with Ad-VLDLR. Intriguingly, expression of the VLDLR in the liver of LDLR$^{-/-}$ mice overexpressing human apoC1 (11/3 × LDLR$^{-/-}$ mice) did not reduce plasma cholesterol

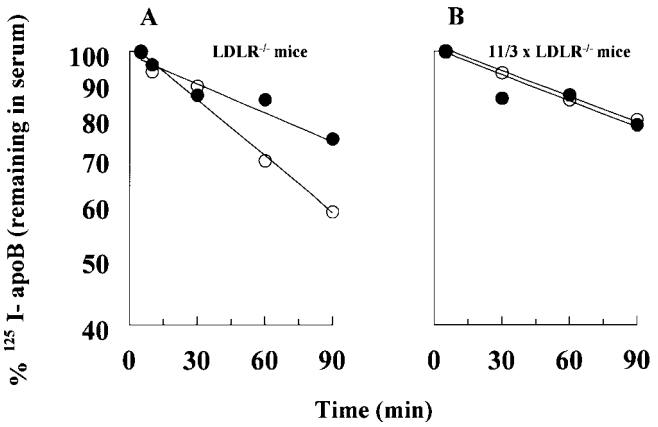

Figure 1 Clearance of apoB in mice injected with Ad-VLDLR and Ad-β-Gal

Fasted LDLR$^{-/-}$ mice (**A**) and 11/3 × LDLR$^{-/-}$ mice (**B**) were injected intravenously with 10 μg of autologous ^{125}I-labelled lipoproteins ($d < 1.019$ g/ml), 5 days after treatment with Ad-VLDLR (○) or Ad-β-Gal (●). Blood was withdrawn 5, 10, 30, 60 and 90 min after injections, and the disappearance of the label was followed in time by measuring ^{125}I-apoB in plasma (see the Materials and methods section). The values shown are expressed as the percentages of radioactivity present after 5 min, and represent the average of two mice per group.

Table 1 Plasma lipid levels in mice injected with Ad-VLDLR, LDLR and β-Gal

Total cholesterol (TC) and TG were measured in the plasma of fasted LDLR-deficient (LDLR$^{-/-}$) *APOC1* transgenic mice (line 11/3, low expresser and 11/1, high expresser) and wild-type mice 5 days after injections with Ad-β-Gal, Ad-VLDLR or Ad-LDLR. All mice were aged 2 months and fed on a regular Chow diet. Values are expressed as the means ± S. D. for at least four mice per group. *$P < 0.05$, indicating the difference between Ad-β-Gal and Ad-VLDLR treatment; †$P < 0.05$, indicating the difference between Ad-β-Gal and Ad-LDLR treatment, all with one strain of mice, using the non-parametric Mann–Whitney test; ‡data shown represent the average for two mice.

	Plasma lipid level (mmol/l)					
	Ad-β-Gal		Ad-VLDLR		Ad-LDLR	
Strain	TC	TG	TC	TG	TC	TG
LDLR$^{-/-}$	10.4 ± 1.2	1.2 ± 0.3	4.3 ± 0.5*	1.1 ± 0.2	3.2 ± 1.4†	0.6 ± 0.5†
11/3 × LDLR$^{-/-}$	36.5 ± 17.3	18.3 ± 13.1	43.5 ± 19.5	21.3 ± 15.1	4.5 ± 1.4†	1.5 ± 0.8†
Wild-type	2.6 ± 0.3	0.2 ± 0.1	1.9‡	0.2‡	1.0 ± 0.5†	0.2 ± 0.2
11/3 (Low expresser)	4.9 ± 0.7	1.3 ± 0.6	4.8 ± 0.6	2.5 ± 1.6	1.8 ± 0.7†	1.2 ± 0.8
11/1 (High expresser)	8.7 ± 3.4	7.5 ± 3.0	8.7 ± 1.5	9.5 ± 2.8	3.1 ± 0.9†	3.4 ± 2.2†

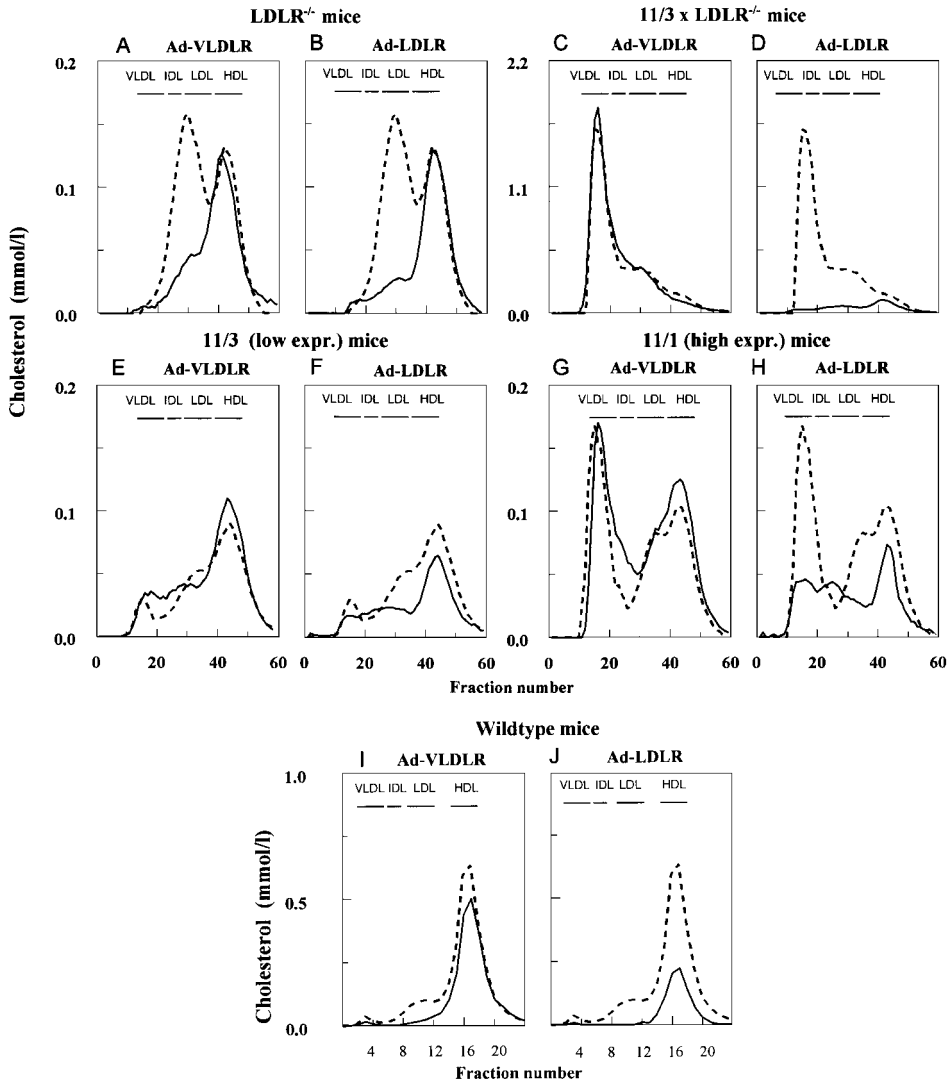

Figure 2 Lipoprotein profiles of mice injected with Ad-β-Gal, Ad-VLDLR and Ad-LDLR

Plasma of LDLR$^{-/-}$ mice (**A**,**B**), 11/3 × LDLR$^{-/-}$ mice (**C**,**D**), *APOC1* transgenic mice of lines 11/3 (**E**,**F**) and 11/1 (**G**,**H**) were pooled and separated on the basis of size by FPLC. Pooled plasma of wild-type mice (**I**,**J**) was analysed using the Smart system as described previously [35]. After Ad-VLDLR administration (continuous lines; **A**,**C**,**E**,**G**,**I**), Ad-LDLR administration (continuous lines; **B**,**D**,**F**,**H**,**J**) and Ad-β-Gal administration (broken lines; **A**–**J**), the total cholesterol content of each individual fraction was measured enzymically, as described in the Materials and methods section. Note the difference in scale of the *y*-axis of (**C**,**D**) and both axes in (**I**,**J**) (run on the Smart System), relative to those of the other parts of the Figure.

levels (Table 1). These results strongly suggest that apoC1 inhibits the binding of lipoprotein particles to the VLDLR *in vivo*. In line with these observations, it is shown in Figure 1(A) that ^{125}I-labelled lipoproteins of the $d < 1.019$ g/ml fraction (representing VLDL/IDL) are cleared more rapidly in Ad-VLDLR-treated LDLR$^{-/-}$ mice than in Ad-β-Gal-treated LDLR$^{-/-}$ mice. In contrast, no effect of VLDLR overexpression on lipoprotein clearance was observed in LDLR$^{-/-}$ mice overexpressing the human *APOC1* gene (Figure 1B). Furthermore, Ad-VLDLR treatment was not able to reverse the hyperlipidaemia in both low- and high-expresser *APOC1* transgenic mice (Table 1, lines 11/3 and 11/1 respectively). Although the number of mice was small, Ad-VLDLR treatment did lower plasma cholesterol levels in wild-type mice (Table 1).

In order to determine whether apoC1 interferes with lipoprotein binding to the LDLR *in vivo*, mice were injected with 3×10^9 PFU of Ad-LDLR. As expected, Ad-LDLR treatment reversed the hyperlipidaemia in LDLR$^{-/-}$ and 11/3 × LDLR$^{-/-}$ mice (Table 1). In contrast with the lack of an effect of VLDLR overexpression on plasma lipid levels in 11/3 and 11/1 mice, additional expression of the LDLR did further reduce plasma cholesterol and TG levels in *APOC1* transgenic mice of lines 11/3 and 11/1 (Table 1). These results thus suggest that apoC1-containing lipoprotein particles are able to bind to the LDLR *in vivo*, whereas they do not bind to the VLDLR when ectopically expressed in the liver. In addition, Ad-LDLR treatment effectively lowered plasma cholesterol levels in wild-type mice (Table 1).

Lipoprotein profiles of Ad-VLDLR- and Ad-LDLR-treated mice

To investigate which lipoprotein fractions were affected after Ad-VLDLR and Ad-LDLR injections in mice, pooled plasma samples of the respective groups were subjected to FPLC analysis

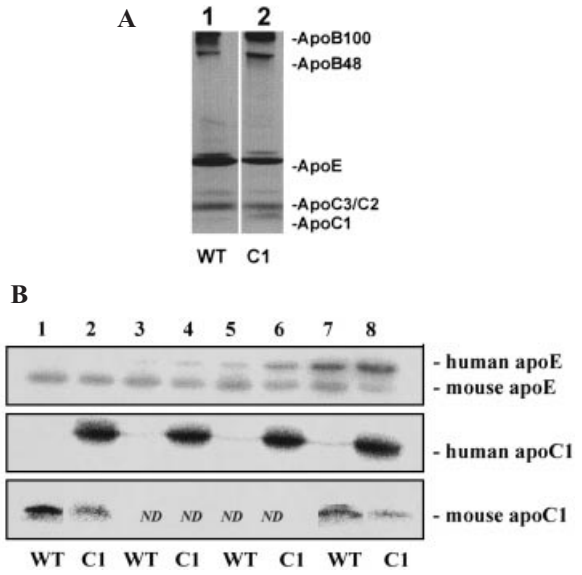

Figure 3 Apolipoprotein composition of wild-type and *APOC1* transgenic VLDL

VLDL ($d < 1.006$ g/ml) was isolated by ultracentrifugation from fasted pooled plasma of Chow-fed wild-type (WT) (**A**,**B**; lane 1) and *APOC1* transgenic mice of line 11/1 (C1) (**A**,**B**; lane 2). (**A**) VLDL (15 μg of protein) was subjected to SDS/PAGE (4–25% gradient gels) and the VLDL proteins were stained with Serva Blue R. (**B**) VLDL fractions were enriched with apoE (**B**, lanes 3–8) and re-isolated by ultracentrifugation, as described in the Materials and methods section. VLDL protein (1.2 μg) was subjected to SDS/PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with polyclonal antisera raised against human and mouse apoE (top panel) and human (middle panel) and mouse apoC1 (bottom panel). As measured with an apoE-sandwich ELISA, wild-type and *APOC1* transgenic VLDL were enriched with 1.4 and 2.4 μg of apoE/mg VLDL protein (**B**, lanes 3 and 4), 3.6 and 5.7 μg of apoE/mg VLDL protein (lanes 5 and 6), and 19.6 and 37.8 μg apoE/mg VLDL protein (lanes 7 and 8) respectively.

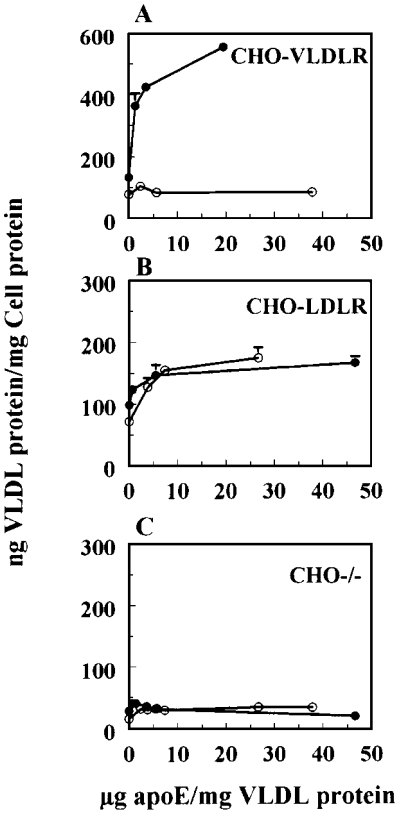

Figure 4 Binding of wild-type and APOC1 transgenic VLDL to CHO cells expressing the VLDLR or LDLR

The binding of VLDL isolated from wild-type mice and *APOC1* transgenic mice to CHO cells expressing the VLDLR (CHO-VLDLR) (**A**), LDLR (CHO-LDLR) (**B**) or lacking both receptors ($CHO^{-/-}$) (**C**) was measured upon incubation of the cells with 10 μg/ml of the respective ^{125}I-labelled VLDL fraction at 4 °C for a period of 3 h. Wild-type VLDL fractions (●) and *APOC1* transgenic VLDL (○) fractions were enriched with the amounts of human apoE plotted along the *x*-axis (see also Figure 3B), before being added to the CHO cells. Binding was determined as described in the Materials and methods section. Values are expressed as ng of VLDL protein/mg cell protein and represent the specific binding (means ± S.D.; $n = 4$). Note the difference in the scale of (**A**) relative to those of (**B**) and (**C**).

with a Superose 6B column. Administration of Ad-VLDLR (Figure 2A) and Ad-LDLR (Figure 2B) to $LDLR^{-/-}$ mice led to a similar decrease in IDL/LDL-sized particles (continuous lines) in comparison with Ad-β-Gal treatment (broken lines). As anticipated from the plasma lipid levels in Table 1, overexpression of the VLDLR in $11/3 \times LDLR^{-/-}$ mice did not alter the lipoprotein profile (Figure 2C). The dramatic lowering of plasma cholesterol levels upon Ad-LDLR administration in $11/3 \times LDLR^{-/-}$ mice was due to a decrease in VLDL/LDL-sized particles (Figure 2D). Similar to $11/3 \times LDLR^{-/-}$ mice, overexpression of the VLDLR in transgenic mice of lines 11/3 (Figure 2E) and 11/1 (Figure 2G) did not significantly change the lipoprotein profiles. The decrease in plasma cholesterol upon Ad-LDLR administration to *APOC1* transgenic mice of lines 11/3 (Figure 2F) and 11/1 (Figure 2H) was mainly confined to the VLDL/LDL-sized fractions. The decreases in plasma TG, as observed after treatment of *APOC1* transgenic mice with Ad-LDLR, was reflected by a lowering of VLDL-sized particles (results not shown). Additional expression of either the VLDLR or the LDLR in the livers of wild-type mice resulted in a lowering of cholesterol in all lipoprotein fractions (Figures 2I and 2J respectively).

Binding of VLDL to CHO cells expressing the VLDLR or the LDLR

To examine further whether the lack of a decrease in cholesterol levels upon overexpression *in vivo* of the VLDLR in *APOC1* transgenic mice was due to an inhibitory action of apoC1 on lipoprotein binding to the VLDLR, *in vitro* binding studies were performed using VLDL ($d < 1.006$ g/ml) isolated from wild-type and high-expresser *APOC1* transgenic mice (line 11/1) with ultracentrifugation. We first analysed the apolipoprotein composition of the respective VLDL particles by means of SDS/PAGE and protein staining. As shown in Figure 3(A), VLDL of high-expresser *APOC1* transgenic mice contained decreased amounts of mouse apoE and increased amounts of apoC1. As calculated, the apoC:apoE mass ratio of *APOC1* transgenic VLDL was elevated about 3-fold when compared with the apoC:apoE mass ratio of VLDL from wild-type mice (1.5 versus 0.5 respectively).

In vitro binding experiments were also performed with VLDL fractions from *APOC1* transgenic and wild-type mice that had been enriched with different amounts of apoE. As shown in Figure 3(B) by Western blot analysis, both *APOC1* transgenic and wild-type VLDL fractions were enriched with increasing amounts of human apoE (Figure 3B, top panel). The amount of endogenous mouse apoE in all *APOC1* transgenic VLDL fractions was decreased, as compared with wild-type VLDL (Figure 3B, top panel). The content of human apoC1 in *APOC1* transgenic VLDL fractions remained unaltered upon addition of

apoE (Figure 3B, bottom panel). Furthermore, the amount of mouse apoC1 on *APOC1* transgenic VLDL was decreased, as compared with wild-type VLDL. Enrichment of the lipoprotein particles with apoE slightly decreased the amount of mouse apoC1 on both wild-type and *APOC1* transgenic VLDL (Figure 3B, bottom panel).

Binding experiments were performed with previously described CHO cells expressing the VLDLR (CHO-VLDLR). For comparison, binding studies were also performed with CHO cells expressing the LDLR (CHO-LDLR) [29]. Binding experiments for 3 h at 4 °C with ^{125}I-labelled VLDL that had not been enriched with apoE showed that both cell types bind less *APOC1* transgenic VLDL as compared with wild-type VLDL (Figure 4). Enrichment of wild-type VLDL with apoE strongly enhanced its uptake by CHO-VLDLR cells in a dose-dependent manner (Figure 4A). Remarkably, enrichment of *APOC1* transgenic VLDL with apoE could not stimulate its binding to CHO-VLDLR cells (Figure 4A), suggesting that apoC1 interferes with the apoE-mediated lipoprotein binding to the VLDLR. Furthermore, enrichment of both *APOC1* transgenic and wild-type VLDL with apoE enhanced lipoprotein binding to the LDLR (Figure 4B) to a similar extent, although the stimulation by addition of apoE was less than that of wild-type VLDL binding to the VLDLR (compare Figure 4A with 4B; note the difference in scale). CHO cells that were negative for both receptors bound little VLDL; this was not affected by enrichment of the particles with human apoE (Figure 4C).

DISCUSSION

In the present study we demonstrate that ectopic overexpression of the VLDLR in livers of *APOC1* transgenic mice does not reduce hyperlipidaemia, whereas it does reverse hyperlipidaemia in LDLR$^{-/-}$ and wild-type mice. That this apparent lack of an effect in *APOC1* transgenic mice was caused by a defective binding of apoC1-enriched lipoproteins to the VLDLR was confirmed by studies both *in vivo* and *in vitro*, showing that (i) the clearance of lipoprotein particles in *APOC1* transgenic mice was not affected upon overexpression of the VLDLR, and (ii) apoC1 inhibited the binding of VLDL to CHO cells expressing the VLDLR. This inhibition was specific for the VLDLR, since overexpression of the LDLR in livers of *APOC1* transgenic mice did lower plasma cholesterol and TG levels in these mice. Thus, whereas the LDLR can clear lipoprotein particles with an excess of apoC1, VLDL clearance, as mediated by the VLDLR, is completely inhibited by apoC1.

As discussed earlier, *in vitro* studies have shown that the VLDLR binds with high affinity to particles containing apoE, such as chylomicrons, β-VLDL and IDL. Recently, we reported that ectopic overexpression of the *VLDLR* gene in the liver of APOE*2 and APOE*3Leiden transgenic mice resulted in a strong decrease in the levels of plasma VLDL-cholesterol and TG [27]. Thus, *in vivo*, the VLDLR recognizes VLDL particles containing these variant forms of apoE. In the present study we show that the VLDLR also clears VLDL/IDL-sized particles circulating in either wild-type or LDLR$^{-/-}$ mice. In contrast, Ad-VLDLR treatment was unable to lower plasma lipid levels in either the hyperlipidaemic low- (line 11/3) or the high-expresser (line 11/1) *APOC1* transgenic mice (Table 1), suggesting that the inhibitory action of apoC1 on lipoprotein binding to the VLDLR is already achieved at moderately elevated levels of human apoC1 in plasma. Since a strong increase in the apoC:apoE mass ratio was observed for VLDL isolated from *APOC1* transgenic mice, it can be postulated that apoC1-enriched lipoprotein particles are defective in binding to the VLDLR, possibly because of their relatively low amount of apoE on the particles. However, additional experiments showed that the defective binding of *APOC1* transgenic VLDL to CHO-VLDLR cells could not be corrected upon enrichment with apoE, whereas apoE enrichment strongly enhanced the binding of wild-type VLDL to these cells (Figure 4A). Since apoE enrichment of *APOC1* transgenic VLDL did not displace human apoC1 from the particle (Figure 3B), we conclude that excess of apoC1 interferes with the apoE-mediated binding of TG-rich lipoproteins to the VLDLR. The experiments reported here do not address whether an excess of apoC1 affects the affinity of the lipoprotein particles for the VLDLR, or whether binding sites on the receptor are being blocked. Previous studies have shown that apoC1 modulates the affinity of the VLDL particle either by displacing apoE from the particle [4] or by changing the conformation of apoE [6]. In the present study, enrichment of *APOC1* transgenic VLDL with apoE was unable to restore the defect in binding of the particles to the VLDLR, suggesting that the inhibitory effect of apoC1 is probably not caused by a massive displacement of apoE, but rather by a masking or a change in the conformation of apoE, that serves as an important ligand for the VLDLR.

Binding of lipoproteins to the LDLR can be mediated by apoE, as well as by apoB. Studies by Clavey et al. [32] showed that apoC1 does not interfere with the apoB-mediated binding of lipoprotein particles to the LDLR, suggesting that *APOC1* transgenic VLDL can still bind to the LDLR *in vivo* via apoB. In the present study we show that although *APOC1* transgenic VLDL exhibits decreased binding to the LDLR *in vitro*, addition of apoE to the *APOC1* transgenic VLDL particle was able to enhance VLDL binding to the LDLR. Thus, from both previous and present data, it can be inferred that particles enriched in apoC1 can bind to the LDLR *in vivo* via apoB and, at least in part, via apoE. In addition, overexpression of the LDLR in wild-type mice strongly decreased HDL-cholesterol levels (Figure 2J). Similar results have been published previously [33] in mice with chronic overexpression of the LDLR, suggesting that low levels of HDLs upon LDLR overexpression are due to an increased receptor-mediated clearance of HDL particles that contain apoE.

Several mechanisms can be proposed to explain the hyperlipidaemia observed in human *APOC1* transgenic mice. First, it has been reported that apoC1 inhibits the uptake of TG-rich lipoproteins by the liver [8,9]. Secondly, we previously reported on elevated levels of FFA in plasma of *APOC1* transgenic mice [10]. Elevated plasma FFA levels might in turn enhance hepatic VLDL production and contribute towards hyperlipidaemia in *APOC1* transgenic mice. In addition, we found that overexpression of RAP, which is a potent inhibitor of lipoprotein binding to members of the LDLR family, did not induce hyperlipidaemia further in 11/3 × LDLR$^{-/-}$ mice, whereas it did in LDLR$^{-/-}$ mice. These results suggest that apoC1 inhibits the clearance of lipoprotein particles via a RAP-sensitive pathway. Since, under these conditions, the clearance of α_2-macroglobulin (as a ligand for LRP) was completely inhibited, we conclude that apoC1 inhibition probably affects the LRP pathway. Recently, liver-specific LRP knock-out mice have been described by Rohlmann et al. [34]. Remarkably, there is a marked distinction between the doubly knock-out (LDLR$^{-/-}$/LRP$^{-/-}$) mice and LDLR$^{-/-}$ mice transfected with Ad-RAP, i.e. LDLR$^{-/-}$/LRP$^{-/-}$ mice accumulate significantly less TG than Ad-RAP-treated LDLR$^{-/-}$ mice do [34]. These data strongly suggest that high circulating levels of RAP, in addition to inhibiting remnant removal via LRP, also interfere with other receptors or metabolic pathways involved in the catabolism of TG-rich lipoproteins. The observation that overexpression of *APOC1* in LDLR$^{-/-}$ mice grossly elevated plasma TG levels (similar to RAP overexpression) strongly

suggests that the hyperlipidaemic phenotype in *APOC1* transgenic mice cannot be attributed solely to LRP blockage by apoC1. Since previous studies [18] have reported that RAP efficiently blocks ligand binding to the VLDLR, and the present study shows that apoC1 is a potent inhibitor of lipoprotein binding to the VLDLR, it can be postulated that the VLDLR might play a role in this pathway. Because of its localization in heart, muscle and fat-cells, the VLDLR is suggested to play a role in the binding of TG-rich lipoproteins to facilitate their peripheral lipolysis, and the subsequent delivery of FFA into these tissues [15,19,20]. Consequently, it is tempting to speculate that the previously reported elevated plasma FFA levels and diminished adipose-tissue mass in *APOC1* transgenic mice might be due (in part) to an impaired interaction between apoC1 transgenic VLDL and the VLDLR. Such a mechanism is supported by the observation that, similar to *APOC1* transgenic mice, mice deficient in the VLDLR exhibit decreased amounts of body fat [21]. Future studies are neccesary to address the postulated role for the VLDLR in the binding of TG-rich lipoproteins and subsequent delivery of FFA to adjacent tissues. Although a physiological role for the VLDLR in TG metabolism has yet to be established, the VLDLR has been proposed as an alternative receptor for lipoprotein clearance in gene-therapy strategies [24,31]; therefore a full characterization of ligands that bind to the VLDLR is necessitated. Assuming that the VLDLR is similarly expressed *in vivo* in humans as it is in mice, the present data show that clearance of lipoproteins can effectively be modulated by apoC1.

In conclusion, whereas apoC1 is reported to inhibit the apoE-dependent interaction of TG-rich lipoproteins with the LRP, the present study shows that apoC1 also hampers the binding of these particles to the VLDLR, as observed both *in vivo* and *in vitro*. Intriguingly, lipoprotein particles enriched with apoC1 are still able to bind to the LDLR. These differences in binding specificity of lipoprotein receptors for apoC1-containing lipoprotein particles might contribute to our understanding of the complex phenotype observed in human *APOC1* transgenic mice.

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