Effect of apolipoprotein A-V on plasma triglyceride, lipoprotein size, and composition in genetically engineered mice

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Abstract **Transgenic (Tg) mice that overexpress the human apolipoprotein A-V gene (*APOA5*) yet lack an endogenous mouse *apoa5* gene (*APOA5* Tg mice) were generated. Subsequently, the effect of human apoA-V expression on plasma triglyceride (TG) concentration and lipoprotein and apolipoprotein distribution was determined and compared with that in mice deficient in apoA-V (*apoa5*$^{-/-}$ mice). NMR analysis of plasma lipoproteins revealed that *APOA5* Tg mice had a very low VLDL concentration (26.4 ± 7.7 nmol/dl), whereas VLDL in *apoa5*$^{-/-}$ mice was 18- fold higher (467 ± 152 nmol/dl). SDS-PAGE analysis of the d < 1.063 g/ml plasma fraction revealed that the apoB-100/apoB-48 ratio was 14-fold higher in *APOA5* Tg versus *apoa5*$^{-/-}$ mice and that the apoE/total apoB ratio was 7-fold greater in *APOA5* Tg versus *apoa5*$^{-/-}$ mice. It is anticipated that a reduction in apoB-100/apoB-48 ratio as well as that for apoE/apoB would impair the uptake of VLDL and remnants in *apoa5*$^{-/-}$ mice, thereby contributing to increased plasma TG levels. The concentration of apoA-V in *APOA5* Tg mice was 12.5 ± 2.9 μg/ml, which is ~50- to 100-fold higher than that reported for normolipidemic humans. ApoA-V was predominantly associated with HDL but was rapidly and efficiently redistributed to apoA-V-deficient VLDL upon incubation. Consistent with findings reported for human subjects, apoA-V concentration was positively correlated with TG levels in normolipidemic *APOA5* Tg mice. It is conceivable that, in a situation in which apoA-V is chronically overexpressed, complex interactions among factors regulating TG homeostasis may result in a positive correlation of apoA-V with TG concentrations.—** Nelbach, L., X. Shu, R. J. Konrad, R. O. Ryan, and T. M. Forte. **Effect of apolipoprotein A-V on plasma triglyceride, lipoprotein size, and composition in genetically engineered mice. *J. Lipid Res.* 2008.** 49: **572–580.**

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Plasma triglyceride (TG) is an independent risk factor in cardiovascular disease and is implicated in the development of the metabolic syndrome, characterized by insulin resistance, hypertension, obesity, and a proinflammatory condition. Apolipoprotein A-V (apoA-V) has been identified as a regulator of plasma TG concentrations in humans and mice. Mice overexpressing apoA-V had significantly reduced (70%) plasma TG concentrations, whereas apoA-V-deficient (*apoa5*$^{-/-}$) mice had an ~4-fold increase in plasma TG compared with control littermates (1). Adenovirus-mediated overexpression of human apoA-V in mice was shown to diminish plasma VLDL-TG levels in a dose-dependent manner (2). Genetic studies in human populations have identified polymorphisms in *APOA5* that correlate with increased plasma TG and risk for cardiovascular disease (3–6). Truncation mutations in *APOA5* have been identified that are associated with severe familial hypertriglyceridemia and chylomicronemia (7, 8), suggesting that apoA-V modulates plasma TG concentrations.

The *APOA5* gene is located in the *APOA1/C3/A4* gene cluster and is expressed only in the liver. Moreover, van der Vliet et al. (9) observed that apoA-V mRNA expression increased by 3-fold during early liver regeneration in the rat. In humans, the circulating concentration of apoA-V is exceedingly low compared with that for other apolipoproteins. Various studies in humans have shown that the mean plasma concentration of apoA-V is between 114 and 258 ng/ml for normolipidemic subjects (10–13). On a molar basis, this suggests that apoA-V levels are ~1,000-fold lower than apoB levels and ~10,000-fold lower than apoA-I concentrations.

A number of studies of human populations with increased TG showed that there is a positive correlation between plasma TG and apoA-V concentrations (14–17).

Abbreviations: apoA-V, apolipoprotein A-V; *apoa5*$^{-/-}$, apolipoprotein A-V-deficient; IDL, intermediate density lipoprotein; PVDF, polyvinylidene difluoride; Tg, transgenic; TG, triglyceride.

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Several studies indicated that plasma apoA-V levels in normolipidemic subjects also displayed a positive correlation between apoA-V and TG (13, 17). The population results would appear to contradict studies of humans expressing apoA-V truncation mutations, in which a deficiency of apoA-V is associated with increased TG (7, 8), and with mouse studies showing a 4-fold increase in TG in *apoa5*$^{-/-}$ mice and a 30% decrease in transgenic (Tg) mice compared with wild-type mice (1). Overall, such results suggest that the role of apoA-V in regulating TG concentrations is complex.

The mechanism whereby apoA-V affects plasma TG levels is not completely understood. It has been suggested that apoA-V may, either directly or indirectly, affect TG hydrolysis and VLDL clearance from plasma or may affect VLDL assembly and secretion at the intracellular level. In vitro studies support the premise that apoA-V affects plasma TG levels extracellularly by increasing the efficiency of lipolysis (2, 18, 19).

In the present study, we generated Tg mice that lack the endogenous mouse apoA-V gene but express the human apoA-V gene (referred to as *APOA5* Tg mice) to determine whether there is an association between apoA-V expression and TG concentration. Because apoB-100 and apoE play key roles in the clearance and catabolism of TG-rich remnants after lipolysis, we also examined the distribution of these apolipoproteins in VLDL and LDL from mice expressing human apoA-V compared with *apoa5*$^{-/-}$ mice. We hypothesized that the extreme differences in plasma TG concentration found in Tg compared with knockout mice may be reflected by changes in apoB and apoE distribution on TG-rich particles.

MATERIALS AND METHODS

Materials

Protease inhibitor cocktail, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (Fluka Pefabloc), gentamycin sulfate, chloramphenicol, Trolox, Tween 20, BSA fraction V (for ELISA), and fatty acid-free BSA were from Sigma-Aldrich. Primary antibodies included affinity-purified polyclonal goat anti-human apoB (International Immunology Corp.), which cross-reacted with mouse apoB, polyclonal rabbit anti-mouse apoE (Biodesign International), polyclonal goat anti-human apoA-V (20), and polyclonal rabbit anti-human apoA-V (10). Secondary antibodies included HRP-conjugated affinity-purified donkey anti-goat IgG and HRP-conjugated affinity-purified goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Novex precast 4–20% acrylamide gradient gels in Tris-glycine buffer were from Invitrogen. Enzymatic assay kits for the determination of plasma lipid components were purchased from Wako Chemicals USA.

Mice

Breeder pairs of *APOA5* Tg and *apoa5*$^{-/-}$ mice were kindly provided by Dr. Len Pennacchio (Lawrence Berkeley National Laboratory) (1). Research on these animals was conducted in conformity with the Public Health Service Policy on the Humane Care and Use of Laboratory Animals. Animals were maintained on a chow diet. All treatments of animals were approved by the Children's Hospital Oakland Research Institute Animal Care and Use Committee. *APOA5* Tg mice were in an FVB strain background and were maintained by crossing with FVB animals (JaxLab). Mice carrying the *apoa5* knockout allele (*apoa5*$^{-/-}$) were obtained as progeny of a second-generation backcross to FVB, although the original knockout allele was obtained with embryonic stem cells of 129/SvJ strain that had been crossed with C57Bl/6 (JaxLab). *Apoa5*$^{-/-}$ mice and their *apoa5*$^{+/+}$ wild-type littermates were identified by PCR of tail DNA as described by Pennacchio et al. (1). Subsequently, knockout mice were backcrossed a third time to the FVB strain. The *APOA5* human Tg mice were bred into the *apoa5*$^{-/-}$ background to obtain mice deficient in endogenous mouse apoA-V and maintained by crossing only to homozygous knockout littermates. In the present study, Tg mice lacking the endogenous mouse *apoa5* gene but expressing the human *APOA5* gene (referred to as *APOA5* Tg mice) were compared with the *apoa5*$^{-/-}$ mice. Only male mice were used.

Plasma collection and lipoprotein isolation

Plasma from male mice, average age ~4 months, fasted for 4 h, was collected into tubes containing K_3EDTA (final concentration, 5 mM) and kept on ice. After centrifugation at 2,000 *g* for 10 min at 4°C, plasma was removed and treated with protective agents, including protease inhibitor cocktail (Calbiochem; Protease Inhibitor Cocktail Set III), 50 μg/ml gentamycin sulfate, 50 μg/ml chloramphenicol, 4 mM EDTA, and 10 μM Trolox. Plasma was stored at 4°C and used within 2 days. VLDL, LDL, and HDL were isolated by sequential ultracentrifugation as described by Lindgren, Jensen, and Hatch (21).

Lipoprotein quantification

Lipoprotein concentrations and sizes were analyzed by NMR spectroscopy (22–24) by LipoScience, Inc. (Raleigh, NC). NMR spectra of plasma pools from four to six mice of each genotype were used to generate VLDL, LDL, and HDL particle concentrations and mean particle diameters and to calculate TG and HDL-cholesterol concentrations (22). Four separate plasma pools of each genotype were analyzed.

SDS-PAGE analysis of lipoproteins

Protein concentrations of lipoprotein fractions were measured by the Markwell modification of the Lowry method (25). To compare amounts of apoB-100, apoB-48, and apoE, SDS-PAGE of lipoprotein ultracentrifugal fractions was carried out using the same amount of protein for each fraction. Gels were stained in Coomassie Blue R-250, and band densities of apolipoproteins were compared using the NIH Image J program (26, 27). Two independent densitometric scans were used to calculate each ratio for each mouse plasma.

Density gradient distribution of apoB-100, apoB-48, apoE, and apoA-V

Cumulative rate flotation centrifugation of plasma pools was carried out according to the method of Wang, Tran, and Yao (28), and 1.0 ml fractions were collected. A constant-volume aliquot from each fraction was analyzed by SDS-PAGE and immunoblotting.

Immunoblotting

Lipoproteins were transferred to polyvinylidene difluoride (PVDF) membranes and probed with primary and secondary antibodies diluted in Tris-buffered saline with 0.05% Tween. HRP-conjugated secondary antibodies were detected using West Femto chemiluminescent substrate (Pierce).

Quantification of plasma apoA-V by ELISA

Sandwich ELISA was performed by a modification of an assay described previously (10). High binding BD Falcon ELISA plates were coated overnight at 4°C with 100 μl/well 1 μg/ml rabbit polyclonal antibody specific for the N-terminal portion of human apoA-V [E. Lilly Research Laboratories (10)] in 100 mM sodium carbonate buffer, pH 9.5. Nonspecific binding sites were blocked using 200 μl/well Pierce SuperBlock in PBS. Antigen samples were diluted in assay buffer, consisting of SuperBlock to which had been added 0.05% Tween-20, 5 mg/ml CHAPS, and 1 mM Pefabloc. Highly purified recombinant apoA-V diluted in buffer and a reference mouse plasma spiked with a known amount of recombinant apoA-V were used to calibrate each assay. Serial 1:3 dilutions of each sample were performed. Detection antibody was HRP-conjugated rabbit polyclonal antibody specific for the C-terminal portion of human apoA-V [E. Lilly Research Laboratories (10)]. Pierce 1-Step Ultra TMB ELISA substrate was used. After 30 min at room temperature, the reaction was stopped with 100 μl/well 2 M H_2SO_4.

Transfer of apoA-V from *APOA5* Tg HDL to *apoa5*$^{-/-}$ VLDL

HDL was ultracentrifugally isolated from pooled plasma from *APOA5* Tg mice, and apoA-V-free VLDL was isolated from pooled *apoa5*$^{-/-}$ mouse plasma. HDL (600 μg of protein containing 0.55 μg of apoA-V) was incubated with apoA-V-free VLDL (300 μg of protein) for 30 min at 37°C. All fractions were in Tris-buffered saline. After incubation, samples were chilled, VLDL and HDL fractions were immediately reisolated by ultracentrifugation (Beckman TL100), and apoA-V was quantified by ELISA.

Statistical analysis

Statistical differences between specific samples were determined by Student's *t*-test. Correlations between apoA-V and TG concentrations were analyzed by Pearson's correlation coefficient. $P \leq 0.05$ was considered statistically significant.

RESULTS

Plasma apoA-V concentration in Tg mice expressing only human apoA-V

Breeding the *APOA5* Tg mouse on the *apoa5*$^{-/-}$ background permits the quantification of human apoA-V without potential interference from endogenous mouse apoA-V. Human population studies revealed a positive correlation between apoA-V and TG in both hyperlipidemic and normolipidemic subjects (14, 17), in contradistinction to the extreme situation in which a deficiency of apoA-V is associated with highly increased TG (7, 8). To assess whether *APOA5* Tg mice might also show a direct association between the plasma concentration of apoA-V and plasma TG concentration, apoA-V was determined by ELISA for a group of 21 *APOA5* Tg mice and correlated with plasma TG concentrations. **Figure 1** shows that apoA-V concentration was positively and significantly ($R^2 = 0.49$, $P = 0.0004$) correlated with TG concentration. When the highly increased TG outlier, as well as the very low TG outlier, were removed from the data set, there was still a strong positive association between apoA-V and TG ($R^2 = 0.558$, $P = 0.013$). ApoA-V concentrations ranged from 9 to 19 μg/ml (mean, 12.5 ± 2.9 μg/ml). The mean human apoA-V concentration in the *APOA5* Tg mouse was thus 50- to 100-fold greater than that reported for endogenous apoA-V in humans (10–13). Within the range of TG values reported here for the *APOA5* Tg mice expressing only human apoA-V, apoA-V values varied directly with TG concentrations.

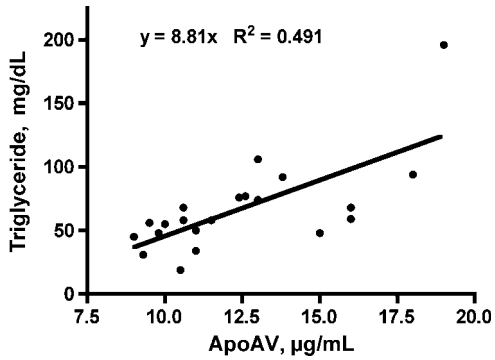

Fig. 1. Relationship between plasma apolipoprotein A-V (apoA-V) and triglyceride (TG) concentrations in *APOA5* transgenic (Tg) mice. Plasma was obtained from *APOA5* Tg mice between 4 and 6 months of age after a 4 h fast. ApoA-V concentration was determined by ELISA on a group of 21 mice. There is a strong positive correlation ($P = 0.0004$) between TG concentration and apoA-V concentration.

Lipid and lipoprotein distribution in *APOA5* Tg and *apoa5*$^{-/-}$ mice

To determine how the expression of the human *APOA5* gene in mice deficient in endogenous apoA-V influences lipoprotein distribution, plasma TG levels, lipoprotein concentrations, and particle sizes were determined using NMR after a 4 h fast. These data were compared with those for *apoa5*$^{-/-}$ mice. As seen in **Fig. 2A**, there was an extremely large difference in VLDL particle concentrations between *APOA5* Tg mice (26.4 ± 7.7 nmol/l) and *apoa5*$^{-/-}$ mice (467 ± 152 nmol/l), where the latter demonstrated an 18-fold increase in concentration. On the other hand, LDL concentration was increased significantly ($P = 0.04$) in *APOA5* Tg mice (311 ± 89 nmol/l) compared with *apoa5*$^{-/-}$ mice (139 ± 120 nmol/l). Plasma TG concentration (822 ± 247 mg/dl) (Fig. 2B), like VLDL concentration in *apoa5*$^{-/-}$ mice, was increased by 18-fold over that of *APOA5* Tg mice (47.6 ± 11.4 mg/dl). It is evident from the data in Fig. 2B that almost all of the TG was associated with VLDL particles in *apoa5*$^{-/-}$ mice, whereas in *APOA5* Tg mice only 50% was associated with VLDL, suggesting that VLDL in the *apoa5*$^{-/-}$ mice was not catabolized normally. The diameter of VLDL particles from *apoa5*$^{-/-}$ mice was 56% larger than that of *APOA5* Tg mice (59.3 vs. 38.0 nm, respectively), whereas LDL size in both groups of mice was the same (Fig. 2C). These data suggest that in the absence of apoA-V, there was not only an increase in concentration of VLDL-sized particles but also the amount of TG per particle increased greatly, thus accounting for the increase in size. The increased size of VLDL seen in *apoa5*$^{-/-}$ mice is in agreement with the observation of Grosskopf et al. (29),

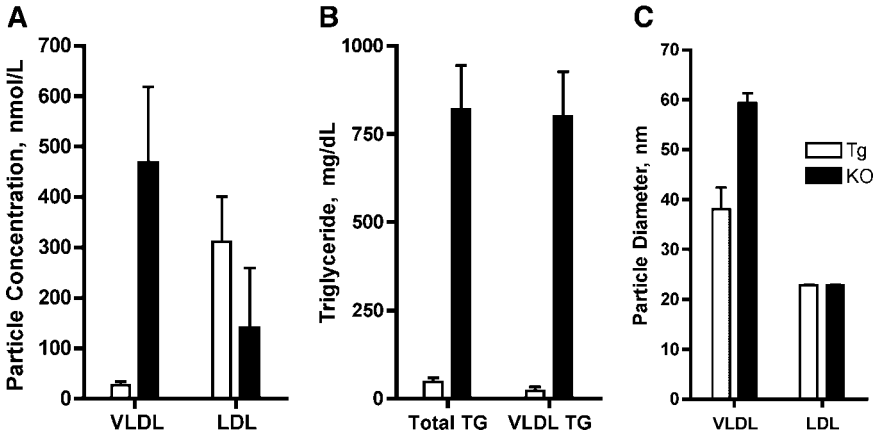

Fig. 2. Plasma VLDL and LDL concentration, TG concentration, and particle size from *APOA5* Tg mice (open bars) and apolipoprotein A-V-deficient (*apoa5*$^{-/-}$) mice (closed bars; KO, knockout) determined by NMR. A: VLDL and LDL particle concentration. B: Total TG versus VLDL TG concentration. C: VLDL and LDL particle size. Plasma pools from four to six mice (average age, 4 months) after a 4 h fast were used. Data reflect means ± SD of four separate experiments.

who noted a 28% increase in VLDL size in *apoa5*$^{-/-}$ mice versus wild-type mice.

Because the large accumulation of TG-rich particles in the *apoa5*$^{-/-}$ mice suggests impaired or delayed lipolysis, we asked whether VLDL and TG concentrations, as well as particle size, normalize with increased time of fast. To address this question, a single experiment using pooled plasma from *apoa5*$^{-/-}$ mice after an 18 h fast was carried out. The concentration of VLDL from *apoa5*$^{-/-}$ mice was reduced by 42% after an 18 h fast, and there was a concomitant 32% reduction in TG concentration. The size of VLDL particles, however, was similar (58 nm) to that of mice fasted for only 4 h, consonant with a reduction in the number of particles. These data are consistent with impaired hydrolysis of VLDL in the *apoa5*$^{-/-}$ mice.

There were no significant differences in HDL particle concentration and HDL-cholesterol concentration between *APOA5* Tg and *apoa5*$^{-/-}$ mice [24.0 ± 3.8 vs. 31.2 ± 8.9 μmol/l ($P = 0.32$) and 86.7 ± 8.4 vs. 87.1 ± 31.1 mg/dl ($P = 0.98$), respectively] after a 4 h fast. HDL particle size, however, was significantly smaller in the *apoa5*$^{-/-}$ mice than in the *APOA5* Tg mice (9.8 ± 0.1 vs. 10.3 ± 0.2 nm; $P = 0.001$).

Distribution of apoB and apoE in the d < 1.063 g/ml plasma fractions of *APOA5* Tg and *apoa5*$^{-/-}$ mice

ApoB-100 and apoE are both recognized by LDL receptor family members and are known to play a key role in the uptake and clearance of VLDL remnants. Because *apoa5*$^{-/-}$ mice possessed large VLDL particles that were slowly cleared from plasma with time, we asked whether the amount of apoB-100 and/or apoE might be altered in the *apoa5*$^{-/-}$ mice compared with *APOA5* Tg mice. The VLDL/LDL (d < 1.063 g/ml) and HDL (d = 1.063–1.21 g/ml) fractions were isolated by ultracentrifugation, and equivalent protein loads were analyzed by SDS-PAGE (**Fig. 3**). Representative data from two experiments indicate that there were major differences in the relative amounts of apoB-100 and apoB-48 between *APOA5* Tg and *apoa5*$^{-/-}$ mice, where apoB-48 was the predominant apoB protein in *apoa5*$^{-/-}$ animals and apoB-100 was predominant in *APOA5* Tg mice (Fig. 3A). There were also major differences in apoE between the two strains of mice, where apoE relative to apoB was clearly increased in *APOA5* Tg compared with *apoa5*$^{-/-}$ mice. **Table 1** summarizes the ratios of apoB-100/apoB-48 and apoE/apoB-100 + apoB-48 obtained by densitometric scan analysis using the NIH Image J program. The ratio of apoB-100 to apoB-48 was considerably greater (14-fold) in *APOA5* Tg mice than in *apoa5*$^{-/-}$ mice, whereas the apoE to total apoB (apoB-100 + apoB-48) ratio was 7-fold greater compared with that in *apoa5*$^{-/-}$ mice. Whereas apoB-100 and apoE levels in the d < 1.063 g/ml fraction from *APOA5* Tg mice showed substantial increases over those of *apoa5*$^{-/-}$ mice, the HDL fraction (d = 1.063–1.21 g/ml) from *APOA5* Tg and *apoa5*$^{-/-}$ mice showed little difference in apolipoprotein content (Fig. 3B).

Distribution of apoB, apoE, and apoA-V in density gradient subfractions of lipoproteins

Cumulative rate flotation centrifugation (28) was carried out to determine whether the distributions of apoB and apoE were affected by the deficiency of apoA-V (**Fig. 4A, B**, respectively). Equivalent volumes of each isolated fraction were electrophoresed by SDS-PAGE and immunoblotted for apoB or apoE. Figure 4A indicates that apoB was distributed throughout the VLDL, intermediate density lipoprotein (IDL), and LDL region in *apoa5*$^{-/-}$ mice, whereas most of the apoB in *APOA5* Tg mice was found in the LDL region, consistent with NMR data. These data also confirm that the major form of apoB in *APOA5* Tg mice was apoB-100, whereas apoB-48 predominated in the *apoa5*$^{-/-}$ mice. ApoE was distributed throughout the VLDL/IDL/LDL density range in both genotypes but was most pronounced in the VLDL

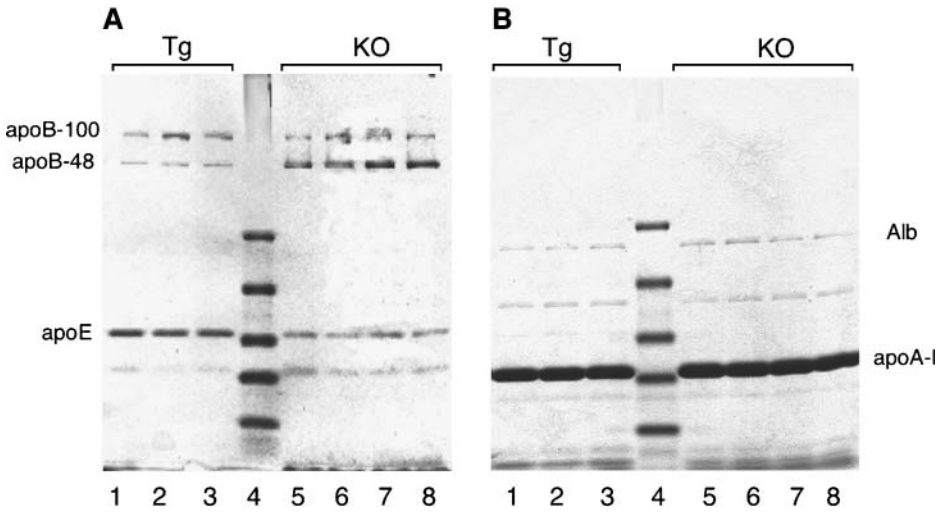

Fig. 3. Representative SDS-PAGE results from the d < 1.063 g/ml fraction (VLDL + LDL) (A) and the d = 1.063–1.21 g/ml fraction (HDL) (B) from *APOA5* Tg or *apoa5*$^{-/-}$ mice. Equal amounts of protein (5 μg) were applied to each lane, and gels were stained with Coomassie Blue R-250. ApoA-V in Tg mouse plasma fractions is not evident because of its low concentration. Alb, albumin; KO, knockout.

fractions of *apoa5*$^{-/-}$ mice. In *APOA5* Tg mice, apoE was most pronounced in the LDL fraction. ApoE was also found throughout the HDL density range but was less abundant in *apoa5*$^{-/-}$ mice than in *APOA5* Tg mice.

The density gradient profile of apoA-V was examined in *APOA5* Tg mice to determine whether apoA-V associated with a distinct class(es) of plasma lipoproteins. As shown in the immunoblot in Fig. 4C, there was a significant amount of apoA-V associated with VLDL/IDL particles (d = 1.007–1.011 g/ml); however, apoA-V was highly enriched and most abundant in HDL particles (note that the HDL fractions were diluted by 20-fold before electrophoresis). Because the most dense fractions of the gradient (d = 1.085–1.101 g/ml) contained plasma proteins as well as HDL, these fractions were pooled, adjusted to d = 1.21 g/ml, and recentrifuged to separate lipoproteins from the lipid-free protein fraction. Both fractions were run on SDS-PAGE gels and probed for apoA-V. ApoA-V was only associated with the lipidated floating fraction (data not shown), indicating that apoA-V was strongly associated with HDL and was not found in the lipid-free form. ApoA-V also colocalized with a distinct subset of LDL particles (d = 1.037–1.063 g/ml) that contained apoE and apoB.

Redistribution of apoA-V from HDL to VLDL

The density gradient result revealing that apoA-V is associated predominantly with HDL suggests that HDL may act as a reservoir for apoA-V, which can transfer to TG-rich particles under appropriate physiological conditions. To test this possibility, HDL was isolated from *APOA5* Tg plasma and incubated with VLDL isolated from *apoa5*$^{-/-}$ mice, and the lipoprotein fractions were reisolated after 30 min. Under the conditions used, in which HDL containing 0.55 μg of apoA-V was incubated with a high concentration of VLDL (300 μg of protein), ELISA quantification revealed that 78% of apoA-V on HDL was transferred to VLDL. This high degree of transfer may reflect the large VLDL surface area available for binding of the hydrophobic apoA-V protein. All fractions were evaluated by SDS-PAGE before and after incubations, and there was no apparent net transfer of other proteins (data not shown).

DISCUSSION

Early studies with apoA-V knockout mice revealed that a deficiency of apoA-V was associated with increased plasma TG (1). The mechanism, however, by which apoA-V affects TG concentrations is not fully understood. Possible mechanisms whereby apoA-V might influence TG levels include the following: *1*) intracellular inhibition of VLDL production in the liver; and *2*) extracellular stimulation of plasma TG hydrolysis, either directly or indirectly by increasing LPL activity, together with accelerated hepatic uptake of VLDL and remnant particles. Although the overexpression of apoA-V in mice, engineered via adenovirus expression vectors, indicated that apoA-V reduced intracellular VLDL lipidation (2), other studies with Tg mice reported that apoA-V has no influence on TG-rich particle production (18, 29, 30). The latter suggests the possibility that in *APOA5* Tg mice, a major, but not exclusive, role of apoA-V

TABLE 1. Ratios for apoB-100/apoB-48 and apoE/apoB-100 + apoB-48 in *APOA5* Tg and *apoa5*$^{-/-}$ mice

Genotype	ApoB-100/ApoB-48	ApoE/ApoB-100 + ApoB-48
apoa5$^{-/-}$ (n = 4)	0.25 ± 0.1	0.32 ± 0.2
APOA5 Tg (n = 3)	3.5 ± 1.6	2.2 ± 1.0

apoa5$^{-/-}$, apolipoprotein A-V-deficient; apoB, apolipoprotein B; Tg, transgenic.

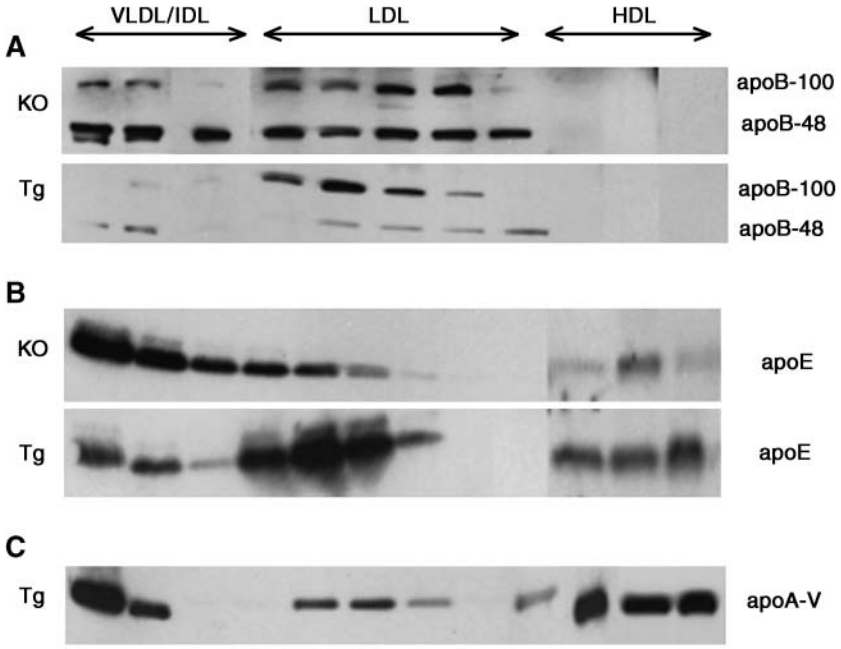

Fig. 4. Subfractions of plasma lipoproteins isolated by cumulative rate flotation centrifugation of plasma from *apoa5*$^{-/-}$ (KO, knockout) and *APOA5* Tg mice. A plasma pool from six mice after a 4 h fast was used; a constant volume (20 μl) from each fraction was analyzed by SDS-PAGE, except for HDL fractions, which were diluted 20-fold before application. The proteins were transferred to polyvinylidene difluoride (PVDF) membranes and probed with antibody for specific proteins. A: Fractions probed for apoB. B: Fractions probed for apoE. C: Fractions from *APOA5* Tg mice probed for apoA-V. A reference tube was used to identify the density of VLDL/intermediate density lipoprotein (IDL), LDL, and HDL fractions.

is the regulation of extracellular events, particularly VLDL TG hydrolysis and the uptake of remnants. Indeed, several studies found that apoA-V increases the efficiency of LPL activity, although it does not directly activate LPL in vitro (18, 19).

In the present study, we found that in *apoa5*$^{-/-}$ mice, VLDL concentration and size were increased greatly over those of *APOA5* Tg mice expressing only human apoA-V, not unlike the results reported for *apoa5*$^{-/-}$ compared with wild-type mice (29). The observed accumulation of these large particles is consistent with a delayed catabolism of VLDL. After a prolonged fast, we found that the number of VLDL particles decreased substantially, consistent with the premise that LPL activity is less efficient in *apoa5*$^{-/-}$ mice, resulting in a slower rate of VLDL lipolysis. The latter confirms the observation of others (19, 29) that the decreased efficiency of LPL activity in the absence of apoA-V contributes to increased plasma TG.

We noted that the amount of apoB-100 relative to apoB-48 in the d < 1.063 g/ml fraction of *apoa5*$^{-/-}$ mice is low compared with that of *APOA5* Tg mice. Because apoB-48, which is not recognized by the LDL receptor, is enriched in VLDL and LDL from *apoa5*$^{-/-}$ mice, uptake of VLDL and its remnants would be impaired. In addition to having less apoB-100, VLDL and LDL from *apoa5*$^{-/-}$ mice had relatively less apoE, where the apoE/apoB-100 + apoB-48 ratio was 0.3 versus 2.2 for *apoa5*$^{-/-}$ versus *APOA5* Tg mice. Because apoE is recognized by both the LDL and LDL receptor-related protein receptors, a reduction in apoE could have significant adverse effects on the clearance of VLDL and its remnants, thus increasing plasma levels of TG. It is interesting that bone marrow transplant studies with apoE-deficient mice, which had grossly increased VLDL and IDL particles enriched in cholesteryl ester, revealed that only small amounts of apoE (<10% plasma concentration) are required for the clearance of VLDL and its remnants (31–33). Although apoB-48-rich VLDL particles in *apoa5*$^{-/-}$ mice are relatively poor in apoE, the bone marrow transplant studies with apoE-deficient mice suggest that the apoE present on the *apoa5*$^{-/-}$ particles should be sufficient for clearance, but clearly this was not the case. It is possible, however, that in the case of *apoa5*$^{-/-}$ mice a critical mass of apoE per VLDL particle is necessary for clearance, but because of the large surface-to-core ratio of these particles, the distribution of apoE and/or its conformation may not be ideal for efficient particle clearance. Grosskopf et al. (29) noted that in *apoa5*$^{-/-}$ mice there was a reduced affinity of VLDL remnants for the LDL receptor. Our observation that apoB-48 is increased and apoE is decreased could in part explain this reduction in affinity and uptake. The shift of apoE from the VLDL fraction in *apoa5*$^{-/-}$ mice to LDL in the *APOA5* Tg mice (Fig. 4) is consistent with previous observations that apoA-V facilitates VLDL hydrolysis (18).

Recent studies by Nilsson et al. (34) using surface plasmon resonance spectroscopy showed that apoA-V can

interact with members of the LDL receptor family, including the LDL receptor-related protein and sortilin-related receptor. Using ligand blotting techniques, Dichlberger et al. (35) demonstrated that lipid-free avian apoA-V also binds to members of the LDL receptor gene family. These observations are interesting in light of our present work, which shows that apoA-V in Tg mouse plasma is present in the LDL as well as the VLDL fractions (Fig. 4). ApoA-V in these fractions could act as a ligand that enhances the binding of LDL and remnant particles to the LDL receptor. This would facilitate the uptake and clearance of these particles.

There was a >20-fold higher level of apoA-V in HDL than in VLDL of *APOA5* Tg mice, suggesting that HDL is a reservoir for apoA-V. This concept is reinforced by the observation that there was a rapid redistribution of apoA-V from HDL to VLDL when *APOA5* Tg HDL was incubated with *apoa5*$^{-/-}$ VLDL. Earlier studies from our laboratory demonstrated that Hep3B cells transiently transfected with human apoA-V also exhibited a preponderance of apoA-V in HDL isolated from conditioned medium (36). The latter report showed that apoA-V did not colocalize with apoB intracellularly but did colocalize with apoB-containing particles in the conditioned medium. Collectively, these data suggest that apoA-V may be secreted on HDL and then transferred to VLDL extracellularly.

In the present study, we show that apoA-V is associated predominantly with the HDL particles and that there appears to be no pool of lipid-free apoA-V, because reisolation of HDL from the protein-rich bottom fraction of the density gradient revealed that apoA-V remained associated with the HDL particle. This is consistent with the previous observations that the protein is extremely hydrophobic and readily binds lipids (37, 38). Truncation studies with apoA-V further revealed that the C-terminal region of the molecule (amino acids 293–343) has lipid binding activity (38). The tight association of human apoA-V with HDL particles is in contrast with a recent report by Dichlberger et al. (35) with the chicken apoA-V homolog, in which a significant fraction of apoA-V was found in the $d > 1.21$ g/ml fraction. The avian apoA-V protein has 42% similarity with the human protein, but there are some major differences in amino acid sequence in the C-terminal region. The latter may account for the differences in lipid association between human and avian apoA-V.

We have shown that, in normolipidemic *APOA5* Tg mice, plasma apoA-V concentrations are positively correlated with TG levels. This observation is surprising because the original studies with *apoa5*$^{-/-}$ mice indicated a 4-fold increase in TG levels compared with littermate controls (1); in contrast, mice expressing the transgene had a 30% decrease in plasma TG. These observations suggested that the presence of apoA-V is associated with a decrease in TG. Mice transfected with apoA-V using an adenovirus delivery system demonstrated a dose-dependent relationship between apoA-V and TG concentrations, in which the delivery of higher concentrations of apoA-V by adenovirus was associated with a greater decrease in plasma TG (2). It may be concluded from these studies that there was an inverse association between TG and apoA-V concentration. The differences in results obtained by adenoviral delivery of apoA-V compared with that of *APOA5* Tg mice, in which there is a positive correlation between apoA-V and TG, may in part reflect differences in the models, because the former is an acute model and the latter is a chronic model. The adenovirus vector without apoA-V has been shown to have an acute response by substantially increasing TG; additionally, the adenovirus delivery of apoA-V is associated with a pronounced decrease in HDL cholesterol (2, 39) not found in the *APOA5* Tg mice. It is interesting that Merkel et al. (18), in in vivo studies on VLDL production in wild-type versus apoA-V Tg mice, noted a trend showing an increase in plasma TG in Tg mice compared with wild-type mice, although the trend did not reach significance. This trend is consistent with our observation that there is a positive correlation between TG and apoA-V in *APOA5* Tg mice.

Human population studies with diabetic and hypertriglyceridemic subjects suggest that plasma apoA-V levels increased, rather than decreased, as TG increased (12, 14–17). The latter observations may be attributable, in part, to other underlying genetic determinants. In several studies, however, a positive correlation between apoA-V and TG concentrations was also reported in normolipidemic subjects (13, 17). The positive correlation between apoA-V and TG concentrations in the current study is the first demonstration that apoA-V and TG variations in a Tg mouse model follow the same trend as those reported for apoA-V in humans.

In summary, no satisfactory explanation currently exists for the apparent contradictions with respect to apoA-V concentration and plasma TG levels. Findings that need to be reconciled include the following: *1*) disruption of the apoA-V gene in mice causes increased plasma TG (1); *2*) overexpression of apoA-V in Tg mice results in decreased plasma TG (1); *3*) adenovirus-mediated overexpression of apoA-V indicates a dose-dependent reduction in plasma TG levels (2); *4*) human population studies with hyperlipidemic and normolipidemic individuals reveal a positive correlation between the plasma concentrations of apoA-V and TG (12–17); and *5*) studies in *APOA5* Tg mice reveal a positive correlation between the plasma concentrations of apoA-V and TG (this study). In considering how each of these distinct findings can be reconciled, it may be useful to consider the effects of insulin on plasma glucose levels. The absence of insulin results in increased glucose, whereas infusion of insulin will induce a decrease and normalization of plasma glucose levels. On the other hand, in the case of insulin resistance, plasma glucose levels are increased despite increased plasma insulin levels. Thus, despite the acute effects of insulin or the lack thereof, in this physiological setting a positive correlation between insulin and glucose exists. In the case of apoA-V and TG, an analogous situation may occur: a complete lack of apoA-V results in increased TG, whereas acute administration of apoA-V (e.g., adenovirus) causes a decline in plasma TG levels. Yet, in a chronic setting or a disease situation, it is conceivable that complex interactions may

lead to a positive correlation between apoA-V and TG levels. Clearly, more work is required to dissect the nature of the complex relationship between apoA-V and TG, but ultimately, it may be possible to explain the seemingly contradictory results that have been generated in different experimental systems.

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