

# Clearance of Cationized LDL Cholesterol From a Muscle Depot Is Not Enhanced in Human Apolipoprotein A-IV Transgenic Mice

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**Abstract**—Human apolipoprotein A-IV (apoA-IV) transgenic mice fed an atherogenic diet were shown previously to develop less atherosclerosis than control mice. The question arose whether the antiatherogenic effect of human apoA-IV is due to enhancement of reverse cholesterol transport despite no increase in plasma high-density lipoprotein (HDL) cholesterol. We studied male and female mice overexpressing human apoA-IV and their wild-type (WT) controls, all of which were fed a chow diet. Plasma total and HDL cholesterol and total phospholipids were not increased in the transgenic mice, and regression analysis showed no correlation between plasma levels of cholesterol or phospholipids and plasma human apoA-IV. To study reverse cholesterol transport in vivo, the disappearance of cholesterol from a depot of [<sup>3</sup>H]cholesterol-labeled cationized low-density lipoprotein injected into the rectus femoris muscle was compared in high expressers of human apoA-IV and WT controls. The loss of radioactivity and the diminution of the exogenous cholesterol mass were determined on days 8 and 12 after injection. No enhanced loss of radioactivity or cholesterol mass was seen in the transgenic mice even at levels of 2500 mg/dL of human apoA-IV. In some instances, there was even slower loss of exogenous cholesterol (radioactivity and mass) in the transgenic mice. Although [<sup>3</sup>H]cholesterol efflux from cultured human skin fibroblasts and mouse peritoneal macrophages was only ≈30% higher in the presence of sera from high expressers of human apoA-IV, addition of phosphatidylcholine liposomes enhanced the efflux in both groups to the same extent. Another paradoxical finding was that the cholesterol esterification rate in plasma was 34% to 36% lower in human apoA-IV mice than in WT controls. In conclusion, even though apoA-IV was found previously to be atheroprotective under hypercholesterolemic conditions, high plasma levels of human apoA-IV did not enhance cholesterol mobilization in vivo in normocholesterolemic mice. (*Arterioscler Thromb Vasc Biol.* 2000;20:179-184.)

**Key Words:** reverse cholesterol transport ■ apolipoprotein A-IV ■ high-density lipoprotein ■ lecithin-cholesterol acyltransferase ■ phospholipids

Apolipoprotein A-IV (apoA-IV) is a 46-kd plasma glycoprotein associated with HDL, but it is found also in the lipoprotein-free fraction of plasma, which was not subjected to ultracentrifugation.<sup>1-4</sup> Studies involving interstitial fluid, as represented by peripheral lymph, have shown the presence of discoid particles that contained phospholipids, free cholesterol, and apolipoproteins, mainly A-I, E, and A-IV.<sup>5</sup> Cholesterol feeding caused a pronounced increase in these particles, some of which were very rich in apoA-IV.<sup>5</sup> The presence of apoA-IV in interstitial fluid suggested its putative role in cholesterol efflux from cells. Indeed, in cell culture, apoA-IV, when complexed to phospholipids, was as effective in cholesterol removal as was apoA-I.<sup>6</sup> These findings were further substantiated when other cell types were used.<sup>7,8</sup> More recently, human apoA-IV transgenic (Tg) mice were pro-

duced in which the transgene was primarily expressed in the intestine and in the liver.<sup>9</sup> Analysis of plasma lipids showed that high expression of human apoA-IV resulted in no change in total cholesterol but elicited a fall in HDL cholesterol (HDL-C) and an increase in VLDL cholesterol. The HDL fraction contained large particles enriched with human apoA-IV.<sup>9</sup> To study the effect of high levels of homologous apoA-IV, Tg mice overexpressing mouse apoA-IV were produced.<sup>10</sup> When these mice were kept on a chow diet, their plasma lipids did not differ from those of controls. Feeding of an atherogenic diet resulted in a rise in total cholesterol and HDL-C in males and females. In mice overexpressing homologous apoA-IV compared with controls, diet-induced aortic lesions were reduced by 30%. Among other parameters studied, there was an increase in stimulation of cholesterol

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efflux from cholesteryl ester-loaded macrophages when HDL fractions isolated from control plasma were compared with those from apoA-IV overexpressers, the plasma of which was derived after mice were fed an atherogenic diet for 2 weeks.<sup>10</sup> A 70% reduction in aortic lesion development was shown in human apoA-IV Tg mice fed an atherogenic diet as well as in human apoA-IV/apoE<sup>-/-</sup> mice kept on a chow diet.<sup>11</sup> Sera derived from human apoA-IV Tg mice fed an atherogenic diet had a 34% higher capacity to promote cholesterol efflux from hepatoma cells.<sup>11</sup> The mechanism of the protective action of human apoA-IV in these mice has not been established, in view of an even more atherogenic lipoprotein profile, especially in the human apoA-IV Tg/apoE<sup>-/-</sup> mice. Therefore, it seemed of interest to study reverse cholesterol transport *in vivo* in human apoA-IV Tg mice and to compare the capacity of their plasma to enhance cholesterol efflux from human and mouse cells in culture. To estimate reverse cholesterol transport *in vivo*, we used a recently developed method that permits quantification of cholesterol efflux from a defined depot.<sup>12</sup> Briefly, 10  $\mu$ L of cationized LDL (cat-LDL) is injected into the leg muscle, and the positively charged lipoprotein is well retained initially and promotes a sterile inflammatory response. The injected lipoprotein is metabolized, as evidenced by hydrolysis of cholesteryl ester and esterification of free cholesterol. By use of this methodology, a delay in cholesterol efflux was found in apoA-I knockout mice compared with controls.<sup>13</sup>

## Methods

### Animals

Tg mice expressing human apoA-IV were generated as described previously.<sup>11</sup> The transgene comprised an 8.4-kb human genomic DNA fragment encoding apoA-IV linked to a 1.7-kb *PstI-PstI* fragment of the hepatic control region of the apoE/C-1 gene.<sup>14</sup> The DNA fragment was injected into one cell embryo of C57/BL6 mice. Two lines of Tg mice expressing human apoA-IV and containing 12 (HuA-IVTg5) and 4 (HuA-IVTg9) copies of the transgene were obtained. In the present study, only HuA-IVTg5 animals were studied. The mice were bred from the retro-orbital plexus to determine the level of human apoA-IV by electroimmunodiffusion and rabbit polyclonal antibodies by a method adapted for human apoA-IV from that described for human apoA-I.<sup>15</sup> There was no cross-reaction between these antibodies and mouse apoA-IV. The mice were reared up to 3 months in France and flown to Jerusalem and kept in a specific pathogen-free facility. Experiments were performed when the mice reached 4 months of age (after an acclimatization period of 1 month). All animals were housed in constant temperature rooms with a 12-hour light-dark cycle and were fed a pelleted chow diet.

### Lipoproteins

LDL was isolated from human plasma according to Havel et al.<sup>16</sup> Cationization of LDL was carried out according to Basu et al.<sup>17</sup> The cat-LDL was dialyzed extensively for 48 hours with frequent changes of dialysis buffer and was labeled with [<sup>3</sup>H]cholesterol, which was added to the lipoprotein by injection of 2 to 4  $\mu$ L of acetone. All preparations used for injection were concentrated to 20 mg cholesterol/mL and were sterilely filtered.

### Experimental Procedure

Injection of cat-LDL was carried out as previously described.<sup>12</sup> Briefly, the animals were anesthetized with peroxide-free ether, the inguinal areas were shaved, and the mice were strapped in a supine position with adhesive tape holding the extremities stretched parallel to the long axis of the body. A longitudinal incision of 3 to 4 mm was made in the skin of the thigh, and 10  $\mu$ L of cat-LDL (200  $\mu$ g

cholesterol) was injected into the rectus femoris muscle with a 1/2-in 27-gauge needle. The needle was kept in place for 1 minute and slowly withdrawn; if a droplet of liquid appeared, it was blotted, and the radioactivity was counted. The incision was closed with 3 or 4 discontinuous sutures. The cat-LDL was injected into the rectus femoris of the right leg, whereas the left muscle was removed when the animals were killed for study for estimation of endogenous cholesterol content. On days 8 and 12 after injection, the animals were anesthetized with ether, blood was drawn from the aorta, and 100 to 150 mg of the rectus femoris muscle on the injected and contralateral side was removed and weighed immediately. The tissue was minced and homogenized in a dual glass conical homogenizer in 2 $\times$ 1 mL of methanol followed with 2 $\times$ 1 mL of chloroform.

### Analytical Procedures

For determination of cholesterol in muscle, stigmaterol was added as an internal standard, and the homogenate was left overnight at room temperature to allow lipid extraction. After centrifugation at 3000 rpm for 10 minutes, the lipid extract was brought to chloroform/methanol (2:1 [vol/vol]) and purified according to Folch et al.<sup>18</sup> Aliquots of chloroform extracts of muscle were taken for determination of radioactivity, for analysis of lipids by thin-layer chromatography, and for determination of total and free cholesterol by high-performance liquid chromatography (HPLC). Separation of [<sup>3</sup>H]cholesterol from [<sup>3</sup>H]cholesteryl ester by thin-layer chromatography was performed with chloroform/ethyl acetate (95:5 [vol/vol]). For determination of cholesterol by HPLC, the chloroform was evaporated, the dry extracts were dissolved in 2-propanol, and the Boehringer reagent for determination of either free or total cholesterol was added. After incubation at 37°C for 45 minutes, methanol was added, the product was extracted with petroleum ether, which was evaporated, and the residue that had been dissolved in 2-propanol was analyzed by HPLC (Kontron 400 equipped with a spectrophotometric detector 430), with the use of an Altech reversed-phase Econosphere C18 5- $\mu$ m 250 $\times$ 4.6-mm column. The product was eluted with acetonitrile/2-propanol (82:18) at a flow rate of 1 mL/min, and the cholesterol and stigmaterol oxidation products were detected at 242 nm.

Estimation of endogenous and exogenous cholesterol in muscle was as follows: total and free cholesterol levels were determined on the noninjected contralateral muscle of each animal and expressed per 100 mg wet weight. The mean of all determinations in each group was designated endogenous cholesterol. The value of exogenous total and free cholesterol in the injected muscle was obtained after subtraction of the endogenous cholesterol.

Total cholesterol and HDL-C were determined by an enzymatic procedure using a Boehringer kit. HDL-C was measured on the supernatant, after dextran sulfate precipitation of whole plasma or serum, by use of a Beckman ultracentrifuge rotor No. TLA 100. Plasma and HDL phospholipids were determined by use of a Sentinel CH kit. Plasma lecithin-cholesterol acyltransferase (LCAT) activity was determined according to Ohta et al.<sup>19</sup> In brief, [<sup>3</sup>H]cholesterol (0.5  $\mu$ Ci) in ethanol was evaporated under N<sub>2</sub> in polystyrene tissue culture wells (Corning). Aliquots of plasma diluted ( $\times$ 5) with PBS were added to the wells and incubated for 16 hours at 4°C and 1 hour at 37°C. The reaction was stopped by immersing in an ice bath. Aliquots were removed for determination of radioactivity, lipids were extracted according to Folch et al.,<sup>18</sup> and free and cholesteryl ester was separated by thin-layer chromatography as described above. Fractional esterification rate was expressed as the difference between percent [<sup>3</sup>H]cholesteryl ester before and after incubation at 37°C. Determination of molar esterification rate was based on the specific activity (dpm/nmol) of free cholesterol in each sample. All determinations were performed in triplicate.

### Cell Culture

#### Human Skin Fibroblasts

Human skin fibroblasts (HSFs) were obtained from volunteers with informed consent and were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% FBS. For each experiment, cells were seeded in 12-well multiwells. To label cells with [<sup>3</sup>H]cholesterol, the latter was added to serum containing medium (1

**TABLE 1. Plasma Cholesterol and Phospholipids in Control (WT) and Human ApoA-IV Tg Mice**

Experiment	Plasma Cholesterol and Phospholipids, mg/dL			
	Males		Females	
	WT	Human ApoA-IV Tg	WT	Human ApoA-IV Tg
1				
Human apoA-IV	...	1085±60	...	1030±70
TC	80±3	84±4	73±3	76±5
HDL-C	54±3	58±3	46±4	41±5
TPL	206±7	210±11	184±6	178±8
2				
Human apoA-IV	...	2106±63	...	2150±130
TC	79±5	82±3	71±4	68±5
HDL-C	52±6	52±5	53±3	43±4
TPL	205±6	220±13	167±7	178±14
HDL-PL	186±11	195±16	144±7	144±16

Values are mean±SE of 10 mice in experiment 1 and 5 mice in experiment 2. TC indicates total cholesterol; TPL, total phospholipid; and PL, phospholipid.

μCi/mL), and the HSFs were grown in the labeled medium from the time of seeding for 10 days.

### Macrophages

Peritoneal macrophages were obtained from C57BL/6 mice 4 days after intraperitoneal injection of thioglycolate.<sup>20</sup> Macrophages ( $2.0 \times 10^6$  per well) were seeded in a 12-well multiwell in 2 mL MEM containing 10% FBS. The nonadherent cells were removed after 2 hours, and the adherent macrophages were cultured for 24 hours in MEM containing 10% FBS. To label cells with [<sup>3</sup>H]cholesterol, the latter was added to serum containing medium (1 μCi/mL). The labeled medium was added to macrophages after removal of nonadherent cells.

### Efflux of [<sup>3</sup>H]Cholesterol From Cultured Cells

To study [<sup>3</sup>H]cholesterol efflux from labeled cells, the medium was removed, and the cells were washed with PBS, with MEM containing 1% BSA, and with serum-free medium at 37°C for 15 minutes each. Thereafter, 0.5 mL of acceptor medium was added, and incubation was carried out for 5 hours for macrophages and 24 hours for HSFs. The acceptor medium consisted of serum-free MEM and serum of human apoA-IV Tg or control mice diluted to 1% or 2% in the culture medium. In some experiments, liposomes of dioleoyl phosphatidylcholine, prepared as described previously,<sup>21</sup> were used.

### Materials

The culture medium and FBS were obtained from GIBCO. [7α(n)-<sup>3</sup>H]cholesterol was from Amersham. All reagents were of analytical grade, and dioleoyl phosphatidylcholine was obtained from Sigma Chemical Co.

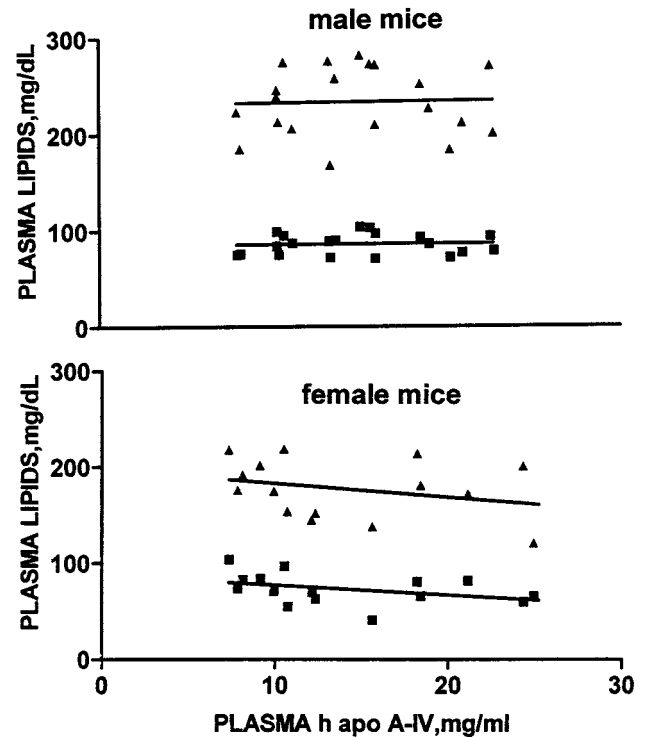
### Statistical Evaluation

The results were presented as mean±SE. The difference between groups was tested with the Student *t* test. Linear regression analysis was performed by using Graph-Pad software.

## Results

### Plasma Lipid Levels

In the present study, the human apoA-IV male and female Tg mice were divided into groups according to their mean levels of plasma human apoA-IV, and their plasma lipid data are presented in Table 1. In experiment 1, the Tg male and female groups had a mean human apoA-IV plasma level of ≈1000



Regression analysis of correlation between plasma levels of human apoA-IV (h apo A-IV) and plasma lipids (triangles indicate phospholipids; squares, cholesterol) in Tg male and female mice.

mg/dL. In males, total cholesterol, HDL-C, and total phospholipids did not differ between WT and human apoA-IV Tg mice. In females, plasma HDL-C levels were ≈10% lower in the human apoA-IV Tg mice compared with WT controls. In experiment 2, the mean human apoA-IV plasma level was ≈2100 mg/dL, and the main difference between the WT and Tg mice was a 20% lower plasma HDL-C level in females. In this experiment, HDL phospholipids were determined as well and accounted for 80% to 90% of total phospholipid in females and males, respectively. Thus, the HDL phospholipid levels in female WT and Tg mice were ≈23% to 26% lower than in male WT and Tg mice, respectively. Regression analysis (Figure) of all plasma values for total cholesterol and phospholipids showed no correlation with increasing levels of plasma human apoA-IV, which ranged between 790 and 2270 and between 730 and 2530 mg/dL in male and female mice, respectively.

### Clearance Of Exogenous Cholesterol (Label and Mass) From Muscle

To determine whether plasma levels of human apoA-IV influence cholesterol clearance from a depot, created by injection of cat-LDL labeled with [<sup>3</sup>H]cholesterol into the muscle, groups of Tg male and female mice were compared with controls (WT) (Table 2). The loss of [<sup>3</sup>H]cholesterol from the depot, as a function of time, was determined by using Tg mice with a mean plasma human apoA-IV level of ≈1000 mg/dL. The clearance of the labeled cholesterol was delayed in both male and female Tg mice compared with WT mice, but the difference did not reach statistical significance (Table 2). Because the loss of [<sup>3</sup>H]cholesterol from muscle represents unesterified cholesterol only, the fate of total

**TABLE 2. Retention of Exogenous Cholesterol (Mass and Label) in Muscle After Injection of Cat-LDL Labeled With [<sup>3</sup>H]Cholesterol**

Day After Injection	Exogenous Cholesterol in Muscle, % of 0 Time			
	[ <sup>3</sup> H]Cholesterol		Cholesterol Mass	
	WT	Human ApoA-IV Tg	WT	Human ApoA-IV Tg
<b>Males</b>				
8	32±2.4	38±2.5	70±4.0	87±4.1*
12	18±1.7	23±1.8	37±3.4	57±4.4*
<b>Females</b>				
8	30±1.5	37±4	71±4.0	79±3.9
12	16±1.7	21±2.0	39±5.0	49±4.0

Values are mean±SE of 5 or 6 males or females for each time point. Tg mice expressing human ApoA-IV (1085±60 and 1030±70 mg/dL in males and females, respectively) and control WT mice were injected in the rectus femoris muscle with cat-LDL (200 µg cholesterol) labeled with [<sup>3</sup>H]free cholesterol. Radioactivity remaining in the muscle after various time intervals was determined on aliquots of homogenates prepared in methanol/chloroform (1:1). To determine the exogenous cholesterol mass, endogenous cholesterol (measured on the contralateral, not injected, muscle) was subtracted from total cholesterol found in the injected muscle.

\**P*<0.05 vs WT cholesterol mass value at corresponding time point.

injected cholesterol mass, of which >70% is in esterified form, was determined as well. As described previously, the cholesteryl ester has to be hydrolyzed before efflux; therefore, the clearance of the injected cholesterol mass has an initial slow component and becomes monoexponential only after the first week.<sup>12</sup> In analogy to the results obtained with labeled free cholesterol, the clearance of exogenous cholesterol mass from the injected muscle of human apoA-IV Tg mice was slower than that of WT controls; however, the difference was statistically significant in males only (Table 2).

The next experiments were designed to determine whether higher plasma levels of human apoA-IV (1600 to 2100 mg/dL) enhances exogenous cholesterol clearance from the depot of [<sup>3</sup>H]cholesterol-labeled cat-LDL. Results presented in Table 3 show that 8 days after injection, there was a tendency for higher retention of cholesterol, mass and label in human apoA-IV Tg mice than in WT controls (statistical significance was found in one experiment), indicating that an increase in plasma apoA-IV did not enhance the loss of exogenous cholesterol.

**TABLE 3. Overexpression of High Levels of Human ApoA-IV Does Not Enhance Loss of Exogenous Cholesterol (Mass and Label) From Muscle After Injection of [<sup>3</sup>H]Cholesterol-Labeled Cat-LDL**

Sex	Human ApoA-IV in Plasma, mg/dL	Exogenous Cholesterol in Muscle, % of 0 Time			
		[ <sup>3</sup> H]Cholesterol		Cholesterol Mass	
		WT	Human ApoA-IV Tg	WT	Human ApoA-IV Tg
Male	1620±55	31±2.0	37±1.8	70±3	86±3*
Male	2106±63	30±0.9	34±2.4	78±3	82±3
Female	2150±130	34±2.4	38±2.0	73±3	81±4

Values are mean±SE of 5 mice in each group on day 8 after injection; other conditions are as in Table 2.

\**P*<0.05 vs corresponding WT cholesterol mass value.

**TABLE 4. Effect of Human ApoA-IV in Mouse Plasma on [<sup>3</sup>H]Cholesterol Efflux From HSFs**

	[ <sup>3</sup> H]Cholesterol Efflux, %/24 h	
	1% Serum	2% Serum
WT serum	9.3±0.3	14.1±0.9
Human apoA-IV		
1520 mg/dL	12.3±0.4*	18.9±0.8†
2050 mg/dL	12.1±0.4*	17.7±0.4†

Values are mean±SE of triplicate wells from 2 experiments. HSFs labeled with [<sup>3</sup>H]cholesterol were incubated for 24 h in medium containing serum obtained from human apoA-IV Tg mice and WT controls. [<sup>3</sup>H]cholesterol efflux indicates <sup>3</sup>H in medium as percentage of total label (sum of cell and medium) after subtraction of efflux to medium without serum.

\**P*<0.05 vs WT serum value (1% serum); †*P*<0.05 vs WT serum value (2% serum).

### Endogenous Cholesterol Esterification in Plasma

Because apoA-IV has been shown to be an activator of LCAT in plasma, in analogy to apoA-I, we also compared cholesterol esterification in plasma derived from human apoA-IV Tg female mice (n=6) and their WT controls (n=6). Contrary to expectations, the fractional esterification rate, expressed as percent of [<sup>3</sup>H]cholesterol esterified after 1 hour of incubation at 37°C, was 7.7±0.6%, and the molar esterification rate was 13.4±1.5 µmol·h<sup>-1</sup>·L<sup>-1</sup> (mean±SE) in human apoA-IV Tg mice. In WT controls, the fractional esterification rate was 12.3±0.9%, and the molar esterification rate was 20.9±1.5 µmol·h<sup>-1</sup>·L<sup>-1</sup>. These differences were significant (*P*<0.01); thus, these values were 37% and 36% lower in the apoA-IV Tg mice than in WT controls. Comparable results were observed on samples of plasma from male mice (3 human apoA-IV and 2 WT mice); data are not shown.

### [<sup>3</sup>H]Cholesterol Efflux From Cultured Cells

To study the capacity of plasma derived from human apoA-IV Tg mice to enhance cholesterol efflux from cultured cells, HSFs labeled with [<sup>3</sup>H]cholesterol were incubated with medium containing 1% or 2% mouse serum (Table 4). It appears that at 1520 mg/dL of human apoA-IV (Table 4), the cholesterol efflux was 32% to 34% higher than in the presence of WT serum, and there was no further increase at the higher apoA-IV concentration. A similar trend was also seen in experiments with mouse peritoneal and J774 macrophages exposed to 2% serum for 5 hours (data not shown). In previous experiments in culture,<sup>6</sup> addition of phospholipid liposomes to purified human apoA-IV resulted in pronounced enhancement of cholesterol efflux from HSFs. Therefore, in the next experiments, the serum used for cholesterol efflux studies was preincubated with increasing concentrations of dioleoyl phosphatidylcholine liposomes (Table 5). It is evident that supplementation of the incubation medium with phosphatidylcholine liposomes enhanced serum-induced [<sup>3</sup>H]cholesterol efflux from macrophages, irrespective of whether the serum was derived from controls or human apoA-IV Tg mice. The effect of phosphatidylcholine liposomes on enhancement of serum-induced [<sup>3</sup>H]cholesterol efflux was also studied in HSFs, and the addition of 0.36 mg phosphatidylcholine/mL increased the efflux 2-fold in both WT and human apoA-IV Tg sera (data not shown).



**TABLE 5. Enhancement of Serum-Induced Efflux of [<sup>3</sup>H]Cholesterol From Macrophages by PC Liposomes**

Experiment	Plasma Human ApoA-IV, mg/dL	PC Liposomes, mg/mL	2% Serum	
			WT	Human ApoA-IV
			[ <sup>3</sup> H]Cholesterol Efflux, %/5 h	
1	1020	None	6.5	7.3
		0.1	9.3	9.7
		0.2	10.3	10.0
		0.4	11.5	11.5
2	2010	None	8.3	10.5
		0.5	13.7	13.1
		1.0	17.2	16.5
		2.0	18.0	19.3

Values are means of duplicate wells that agreed within 10% from 2 experiments. Thioglycolate-elicited mouse peritoneal macrophages, labeled with [<sup>3</sup>H]cholesterol, were incubated for 5 h in medium containing 2% serum obtained from control (WT) or human apoA-IV Tg mice, in absence or presence of increasing concentrations of phosphatidylcholine (PC) liposomes.

### Discussion

The aim of the present study was to determine the effect of high levels of human apoA-IV on cholesterol efflux in vivo in Tg mice. Results obtained in a previous study<sup>22</sup> in human apoA-I Tg mice have shown no enhancement of clearance of cat-LDL cholesterol from muscle even with plasma human apoA-I levels of 308 ± 10 and 429 ± 18 mg/dL in females and males, respectively. The lack of increase in cholesterol clearance from the depot in human apoA-IV Tg mice could be explained in part by kinetic studies in hamsters<sup>23</sup> and mice.<sup>24</sup> The latter study has shown that reverse cholesterol transport in vivo, as represented by selective receptor-mediated uptake of HDL cholesteryl ester by the liver, is saturated at normal HDL concentrations and does not increase even at apoA-I levels 10-fold higher than normal.<sup>23</sup>

There are some differences between apoA-I and apoA-IV in plasma; the more important is the presence of a large fraction of apoA-IV in nonlipoprotein form.<sup>1-4</sup> In Tg mice, the expression of human apoA-I results in a significant increase in plasma total cholesterol, HDL-C, and phospholipids,<sup>22,25</sup> but in human apoA-IV Tg mice, as reported in previous studies<sup>9,11</sup> and in the present study, no change in plasma levels of total cholesterol was seen. HDL-C was not increased in human apoA-IV Tg mice; this finding was similar to that in other studies.<sup>9,11</sup> Moreover, no increase in plasma lipids was seen in mice overexpressing mouse apoA-IV.<sup>10</sup> It seems pertinent to stress that expression of high levels of human apoA-IV was not accompanied by a commensurate increase of plasma phospholipids. In human apoA-I Tg mice,<sup>22</sup> there was a 60% to 85% increase in plasma phospholipid, but the enrichment in apoA-I was relatively much higher and therefore the phospholipid/apoA-I ratio was significantly lower than in controls, contributing in part to lack of enhancement of cholesterol efflux in vivo.<sup>22</sup> In the present study in human apoA-IV Tg mice, there was not only no enhancement but even a slight retardation of lipoprotein cholesterol clearance from the injected depot, which might

have been related to the lipid-poor apoA-IV that was due to no increase in plasma phospholipid. Studies in cell culture<sup>6</sup> provided evidence that apoA-IV became an efficient cholesterol acceptor from cells only if complexed to phospholipid.

Another puzzling finding was that endogenous esterification of [<sup>3</sup>H]cholesterol in the plasma of human apoA-IV Tg mice not only did not increase but was approximately 36% lower than in WT controls. Similar observations were made when rat apoA-IV was added to rat plasma and caused a modest but statistically significant decrease in cholesterol esterification.<sup>26</sup> Before that finding, it was reported that excess of apoA-I added to phosphatidylcholine-cholesterol complexes also decreased the LCAT-dependent cholesterol esterification.<sup>27</sup> These results suggest that the inhibition of cholesterol esterification induced by apoA-IV is due to competition between HDL associated apoA-I with the added apoA-IV for the LCAT binding site.<sup>26</sup> In this conjunction, it is pertinent to note that apoA-IV using natural substrates is a less efficient activator of LCAT than apoA-I.<sup>28-30</sup> Taken together, it seems plausible that the very high concentration of human apoA-IV in the Tg mice (150-fold of normal human levels) affected LCAT activation. Because LCAT is apparently involved in reverse cholesterol transport,<sup>31,32</sup> its lower activity in the Tg mice might have contributed toward the less effective clearance of the injected cholesterol from the depot. Cholesterol efflux from [<sup>3</sup>H]cholesterol-labeled cells was not affected by the lower activity of LCAT in the human apoA-IV Tg mouse plasma, because LCAT activity is not required for the initial step of cholesterol efflux from cultured cells.<sup>33,34</sup> It should be pointed out that in Tg mice fed an atherogenic diet for 2 weeks, a 3- to 6-fold overexpression of mouse apoA-IV resulted in a significant increase in endogenous cholesterol esterification rate in plasma.<sup>10</sup>

How do the presently reported findings relate to the atheroprotective capacity of apoA-IV in Tg mice expressing either human<sup>11</sup> or mouse<sup>10</sup> apoA-IV? Although no increase of clearance of cat-LDL from muscle of human apoA-IV Tg mice was observed in the present study, it does not preclude the role of apoA-IV in reverse cholesterol transport in mice. This role may occur when additional stress conditions that are induced genetically (like apoE-deficient background) or by feeding a high-fat high-cholesterol diet are present. In a previous publication,<sup>11</sup> the authors concluded that human apoA-IV appears to protect Tg mice against atherosclerosis by a mechanism that does not involve an increase in HDL concentration. The atheroprotective property of HDL has also been ascribed to HDL-associated paraoxonase, which acts as an antioxidant.<sup>35</sup> Indeed, an increased serum paraoxonase activity was detected in the mouse apoA-IV Tg mice.<sup>10</sup> More recently, it has been shown that apoA-IV itself is a potent antioxidant produced by the small intestine to protect against lipoprotein oxidation.<sup>36</sup> The antioxidant activity of apoA-IV exceeds that of apoE, and the authors<sup>36</sup> suggest that the protection against development of atherosclerosis observed in apoE knockout mice crossbred with human apoA-IV Tg mice<sup>11</sup> was due to the antioxidant property of apoA-IV. So far, the atheroprotective activity of apoA-IV and of apoA-I has been confined to prevention of atherosclerosis, except for a recent report,<sup>37</sup> in which injection of recombinant adenovirus encoding human apoA-I resulted in regression of atherosclerotic lesions in LDL receptor-deficient mice.

In conclusion, the model system used at the present time (ie, removal of cholesterol from an exogenously introduced lipoprotein depot) could simulate, to some extent, conditions prevailing during regression of atheroma. Therefore, it seems appropriate to point out that the role of high levels of apoA-IV in regression of atherosclerosis remains to be established.

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