short communication

Apolipoprotein A-V association with intracellular lipid droplets[S]

Xiao Shu,[*,†] Joanne Chan,[*] Robert O. Ryan,[*,†] and Trudy M. Forte[1,*]

Center for Prevention of Obesity, Diabetes, and Cardiovascular Diseases,* Children's Hospital Oakland Research Institute, Oakland, CA 94609; and Department of Nutritional Sciences and Toxicology,† University of California, Berkeley, CA 94720

Abstract Apolipoprotein A-V (apoA-V) plays a key role in the regulation of triglyceride (TG) metabolism. Given the very low concentration of apoA-V in plasma, we hypothesized that apoA-V may influence plasma TG levels by affecting the assembly and/or secretion of apoB-containing lipoproteins. When apoA-V was overexpressed in cultured Hep3B cells, neither the amount of apoB secreted nor the density distribution of apoB-containing lipoproteins was affected. Fluorescence microscopy and cell lysate immunoprecipitation studies revealed that apoA-V is not associated with apoB intracellularly, yet immunoprecipitation of apoA-V from the cell culture medium resulted in coprecipitation of apoB. These data suggest that the apoA-V association with apoB-containing lipoproteins is a postsecretory event. Confocal fluorescence microscopy revealed the presence of apoA-V in distinct cellular structures. Based on Nile Red staining, we identified these structures to be intracellular lipid droplets. These data suggest that apoA-V has a unique association with cellular lipids and, therefore, may be involved in the storage or mobilization of intracellular lipids.—Shu, X., J. Chan, R. O. Ryan, and T. M. Forte. **Apolipoprotein A-V association with intracellular lipid droplets. *J. Lipid Res.* 2007.** 48: **1445–1450.**

Supplementary key words Hep3B hepatoma cell line • triglyceride • very low density lipoprotein • immunoprecipitation • confocal fluorescence microscopy • apolipoprotein B

Epidemiological studies have revealed that increased plasma triglyceride (TG) represents an independent risk factor for coronary heart disease (1). Apolipoprotein A-V (apoA-V), a protein expressed solely in the liver, was shown to be involved in TG metabolism in rodents (2, 3). ApoA-V is synthesized with a cleavable signal peptide, suggesting that it will be directed toward a secretory pathway. The concentration of apoA-V in human plasma, however, is extremely low (24–406 ng/ml) (4) compared with other apolipoproteins, such as apoA-I (~1 mg/ml) or apoC-III (~0.1 mg/ml), bringing into question the functional role of plasma apoA-V.

Compared with control littermates, the concentration of TG was increased in homozygous apoA-V knockout mice and decreased in human apoA-V transgenic mice (2). The TG-lowering effect of apoA-V in mice was confirmed by van der Vliet et al. (5) using adenoviral overexpression of mouse apoA-V. Population studies of *APOAV* single nucleotide polymorphisms (2, 6–8) and truncated apoA-V variants (9, 10) have provided support for the hypothesis that apoA-V influences human plasma TG levels.

The mechanism whereby apoA-V decreases TG is not completely understood, but it has been suggested that apoA-V may enhance LPL activity, either directly or indirectly (11–13). Considering the extremely low concentration of apoA-V in the circulation, however, it is conceivable that the metabolic role for apoA-V is intracellular rather than extracellular. Using apoA-V transfected COS-1 cells, Weinberg et al. (14) found that, compared with human serum albumin or apoB-6.6, apoA-V is largely retained in the cell. An intracellular function for apoA-V was suggested by Schaap et al. (12), who found that adenovirus-mediated apoA-V expression in mice decreases VLDL-TG production rate in a dose-dependent manner yet has no effect on VLDL particle number, suggesting that apoA-V impairs the lipidation of apoB but does not impair its secretion. Finally, the observation by van der Vliet et al. (3) that apoA-V mRNA is upregulated during liver regeneration suggests that apoA-V serves a function in hepatocyte proliferation. Using the human liver cell line Hep3B, we examined the effect of apoA-V expression on apoB-100 secretion and lipidation. Surprisingly, we found that apoA-V does not colocalize with apoB intracellularly but, rather, can be found in association with cytosolic lipid droplets.

Abbreviations: apoA-V, apolipoprotein A-V; GFP, green fluorescent protein; Met/Cys, methionine/cysteine; OA, oleic acid; TG, triglyceride.

[1] To whom correspondence should be addressed.
e-mail: tforte@chori.org

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MATERIALS AND METHODS

Materials

[^{35}S]methionine/cysteine (Met/Cys) was purchased from GE Healthcare. Oleic acid (OA), albumin, monoclonal anti-FLAG M2 antibody, and anti-FLAG M2 affinity gel were from Sigma. MEM, DMEM without Met and Cys, sodium pyruvate solution, MEM nonessential amino acid solution, FBS, horse serum, G418, and trypsin-EDTA were purchased from Gibco. Fluorescence-labeled goat anti-mouse Alexa Fluor 594, Nile Red, and 4′,6-diamino-phenylindole (DAPI) were from Molecular Probes. Antibodies used included polyclonal goat anti-apoA-V(15), monoclonal mouse anti-apoE 1D7 (a gift from Dr. Karl Weisgraber), anti-human apoB monoclonal antibody, 1D1, which recognizes only human apoB (University of Ottawa Heart Institute), polyclonal goat anti-apoB (International Immunology Corp.), and monoclonal mouse anti-apoA-I (Intracel).

Cell culture

The human hepatocarcinoma cell line Hep3B (American Type Culture Collection) was cultured in MEM containing 10% FBS, 1 mM sodium pyruvate, and 100 μM nonessential amino acids. Rat hepatoma McA-RH7777-A18 cells stably transfected with human apoB-100 (kindly provided by Dr. Zemin Yao) were cultured in DMEM containing 10% FBS, 10% horse serum, and 200 μg/ml G418. Cells were passaged every 4 days. Cells were transfected using Lipofectamine 2000 (Invitrogen).

Construction of plasmids

To obtain a FLAG-tagged apoA-V (apoA-V-FLAG) expression vector and apoA-V-green fluorescent protein (GFP) fusion protein expression vector, PCR was carried out using a plasmid harboring the entire apoA-V coding region (a gift from Dr. Len Pennacchio). The amplification product for the FLAG tag was cloned into pFLAG-CMV-5.1 (Sigma) and that of GFP was cloned into pEGFP-N1 (Clontech) via *Hind*III and *Bam*HI sites, respectively. Both tags were appended to the C terminus of the protein.

Immunoprecipitation and immunoblotting

Collected cells were washed with cold phosphate-buffered saline and subsequently lysed in a nondenaturing lysis buffer described by Beckstead et al. (15). Immunoprecipitation was carried out according to Wu et al. (16). Protein samples were electrophoresed and immunoblots processed as described previously (17).

Pulse-chase experiments

Cells were incubated in Met/Cys-deficient DMEM for 1 h, then pulsed with [^{35}S]Met/Cys (200 μCi/ml) in Met/Cys-free DMEM containing 10% FBS and 0.8 mM OA for 20 min followed by a 3 h chase. Conditioned medium was collected and subjected to cumulative rate flotation centrifugation (18). One milliliter fractions were collected, immunoprecipitated, and analyzed by SDS-PAGE followed by autoradiography.

Confocal microscopy

McA-RH7777-A18 cells were grown on poly-L-lysine coverslips (BD Biosciences). After transfection with apoA-V-GFP or apoA-V-FLAG, cells were transferred to growth medium supplemented with 0.8 mM OA for 6 h. For immunocytochemistry, cells were fixed with 4% paraformaldehyde in PBS and processed as described (18). Anti-human apoB monoclonal antibody 1D1 was diluted 1:500 in blocking solution and incubated with the cells for 1 h. Cells were washed with PBS and incubated with Alexa Fluor 594-labeled goat anti-mouse IgG. For lipid droplet staining, a Nile Red-saturated (19) acetone solution was diluted 1:100,000 in PBS and incubated with cells for 15 min. Images were captured by a LSM 510 Meta ultraviolet/visible confocal microscope.

RESULTS

Effect of apoA-V expression on apolipoprotein accumulation in cells and medium

Hep3B cells were transfected with a control empty vector or apoA-V plasmid construct. Immunoblot experiments (**Fig. 1**) with the indicated antibodies were performed to determine the relative distribution of apolipoprotein in cell lysates versus conditioned medium. In Hep3B cells transfected with an empty vector, apoA-V could not be detected, consistent with the very low concentration of this protein reported by others (4). In apoA-V transfected cells, however, the protein was readily detected, with roughly similar amounts present in cell lysate and medium. By contrast, nearly all of the apoA-I and most of the apoE and apoB-100 detected was in the medium. The similar apolipoprotein distribution seen in control vector and apoA-V transfected cells indicates that overexpression of apoA-V does not affect the secretion efficiency of these apolipoproteins. At the same time, however, the difference in apolipoprotein distribution between apoA-V and the other apolipoproteins suggests that its secretion is impaired.

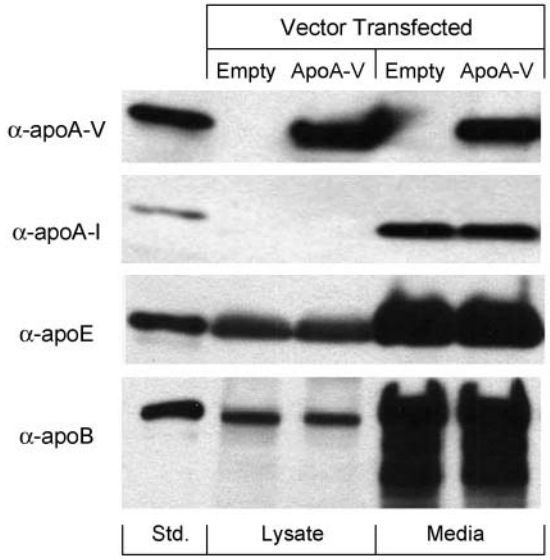

Fig. 1. Effect of apolipoprotein A-V (apoA-V) expression on apolipoprotein accumulation in cells and medium. ApoA-V expression vector and empty vector were transfected into Hep3B cells. Conditioned medium was collected and concentrated to the same volume as cell lysates. Equivalent volume loads of cell lysate and conditioned medium were subjected to SDS-PAGE followed by Western blotting for apoA-V, apoA-I, apoE, and apoB. The apolipoprotein standards (Std.; 20 ng) apoA-V, apoA-I, apoE, and apoB (as human LDL) are indicated at left. The data presented are representative of three independent experiments.

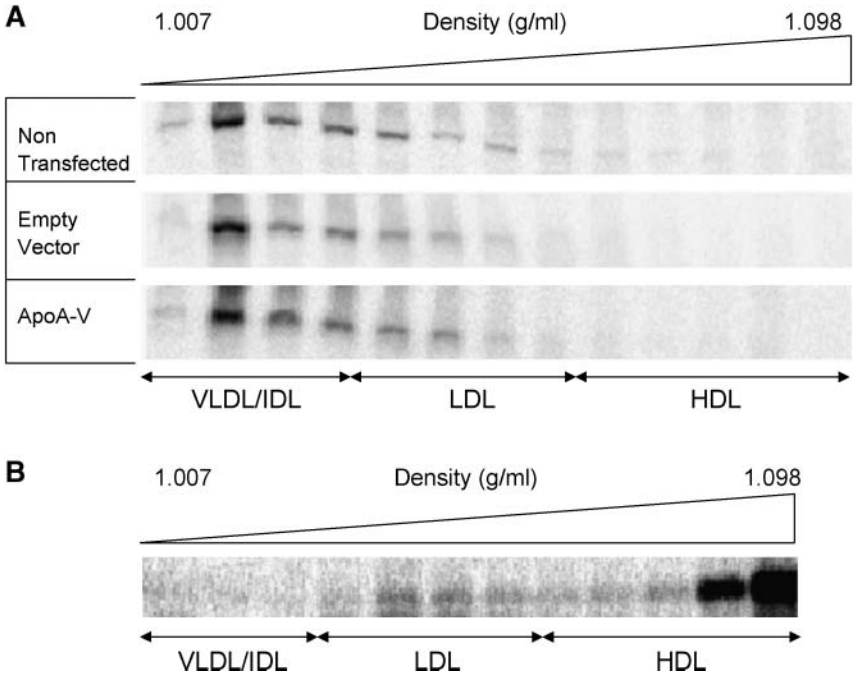

Fig. 2. Effect of apoA-V expression on the density distribution of apoB- and apoA-V-containing lipoproteins isolated from Hep3B medium. Hep3B cell cultures transfected with the apoA-V-FLAG expression vector were pulse-labeled with [^{35}S]methionine/cysteine (Met/Cys) for 20 min followed by a 3 h chase in the presence of 0.8 mM oleic acid (OA). Conditioned medium was collected and subjected to cumulative rate flotation centrifugation. Consecutive 1 ml fractions were collected and immunoprecipitated with apoB antibody (A) or anti-FLAG antibody (B) for apoA-V and analyzed by SDS-PAGE followed by autoradiography. IDL, intermediate density lipoprotein.

Effect of apoA-V expression on the density distribution of apoB- and apoA-V-containing lipoproteins

In murine models, apoA-V exerts a strong influence on plasma TG levels. Thus, it is conceivable that, although apoB-100 secretion from Hep3B cells is unaffected by apoA-V transfection, TG-rich lipoprotein particle density distribution may be altered. To examine this possibility, conditioned medium from [^{35}S]Met/Cys-labeled Hep3B cell cultures was subjected to cumulative rate flotation centrifugation followed by immunoprecipitation with an antibody directed against apoB-100 (**Fig. 2A**). In the case of nontransfected Hep3B cells, apoB is recovered mainly in the VLDL/IDL density range, with a small quantity in LDL. A similar apoB distribution was observed in cell cultures transfected with empty vector or apoA-V vector. We conclude that apoA-V does not influence the lipidation state of apoB secreted from these cells.

Immunoprecipitation of apoA-V (Fig. 2B) indicated that apoA-V is associated primarily with particles in the HDL density range. A small amount of apoA-V is found in LDL and VLDL.

ApoA-V is associated with apoB-containing lipoproteins in the medium but not in cell lysate

In Hep3B cell medium, apoA-V was present in the VLDL/LDL density range; therefore, we asked whether apoA-V and apoB are localized on the same lipoprotein particles. Anti-FLAG pulled down apoA-V and apoB from the medium, and immunoprecipitation with anti-apoB likewise pulled down apoB and apoA-V (**Fig. 3**, right panels). The latter confirms that apoA-V associates with apoB extracellularly and is consistent with the observation

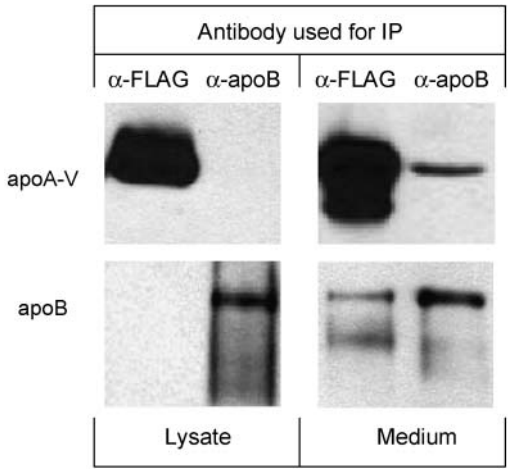

Fig. 3. Apolipoprotein immunoprecipitation (IP) from apoA-V transfected cells. ApoA-V-FLAG was transfected into Hep3B cells. Conditioned medium and cell lysate were immunoprecipitated with either anti-FLAG or anti-apoB antibody. Precipitates were analyzed by SDS-PAGE followed by Western blotting with antibodies to apoA-V and apoB as indicated. Left panel, cell lysate; right panel, conditioned medium.

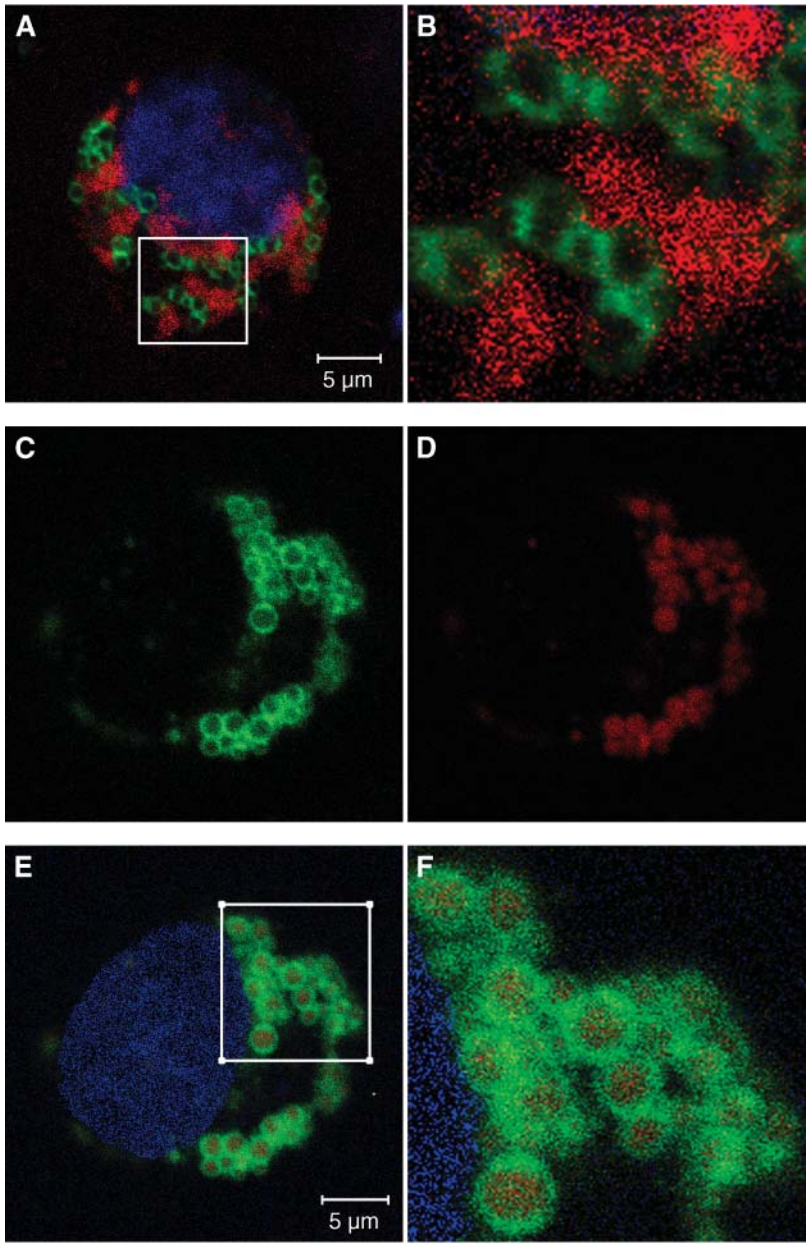

Fig. 4. Confocal fluorescence microscopy images of apoA-V-green fluorescent protein (GFP), human apoB, and lipid droplets in McA-RH7777 cells. Human apoB-100-expressing McA-RH7777 cells transfected with apoA-V-GFP were incubated in the presence of 0.8 mM OA for 6 h, fixed, and permeabilized. A shows a merged image in which apoB was probed with Alexa Fluor 594-labeled secondary antibody (red fluorescence) and apoA-V-GFP (green); B is an enlarged image of the boxed area in A. C and D show apoA-V-GFP (green) and lipid droplets stained with Nile Red (red fluorescence), respectively. E presents a merged image of apoA-V-GFP and Nile Red staining; an enlarged image of the boxed area is shown in F. Cell nuclei were stained with DAPI (blue) in A and E.

that apoA-V is found on VLDL in plasma (4). Hep3B cells expressing apoA-V-FLAG were lysed and the lysate was immunoprecipitated with both anti-FLAG and anti-apoB antibodies. Anti-FLAG antibody pulled down apoA-V but not apoB from the lysate (Fig. 3, left panels); similarly, apoB antibody pulled down apoB but not apoA-V, suggesting that intracellularly, apoB and apoA-V are not associated.

ApoA-V and apoB localize to separate subcellular compartments

Because Hep3B cells have low levels of intracellular apoB (Fig. 1), McA-RH7777 cells expressing high levels of human apoB-100 were transfected with an apoA-V-GFP vector and used to assess whether apoA-V and apoB-100 traffic together intracellularly. Confocal images (**Fig. 4**) of fixed cells probed with anti-apoB followed by an Alexa

Fluor 594-labeled secondary antibody confirmed the localization of this apolipoprotein to the endoplasmic reticulum (18). On the other hand, apoA-V-GFP displayed a very different distribution pattern, clustering around distinct cytosolic structures that display a doughnut-shaped appearance with nonstaining cores. No colocalization of apoB and apoA-V was observed (Fig. 4A, B) consistent with the immunoprecipitation studies with cell lysate. Based on the distribution of apoA-V and its localization to cytosolic spherical entities, we hypothesized that apoA-V may be associated with intracellular lipid droplets. To test this, lipid droplets were stained with Nile Red and apoA-V was monitored by GFP fluorescence (Fig. 4C–F). As seen in Fig. 4C, apoA-V localized to the perimeter of lipid droplets that stain with Nile Red (Fig. 4D). The merged images shown in Fig. 4E, F confirm the colocalization of apoA-V and intracellular lipid droplets. Control studies with native apoA-V and FLAG-tagged apoA-V (see supplementary Figure I) indicated that the observed localization was not attributable to the GFP tag. Cells transfected with apoA-I-GFP (see supplementary Figure II) revealed that, unlike apoA-V, this protein did not localize to lipid droplets but was associated with the endoplasmic reticulum. These data suggest specificity of apoA-V for lipid droplets.

DISCUSSION

Evidence obtained from genetically engineered mice (2) and human population studies (4, 9) has revealed that apoA-V plays a role in modulating TG levels. The mechanism whereby apoA-V regulates TG metabolism is not fully understood. One body of evidence suggested that apoA-V functions extracellularly by activating lipoprotein lipase, thereby increasing the efficiency of chylomicron-TG and VLDL-TG clearance (12, 13, 20). Given the low concentration of apoA-V in plasma, it is estimated that only a small subfraction of circulating VLDL particles will contain an apoA-V molecule (21). This raises the possibility that apoA-V's effects on plasma TG may be intracellular rather than in the plasma compartment.

In this study, we show that apoA-V secretion from cultured Hep3B cells is impaired but that its overexpression has no effect on apoB secretion or lipidation. This observation is consistent with that of Weinberg et al. (14), who noted that apoA-V expressed in COS-1 cells had a low secretion efficiency compared with albumin and a truncated form of human apoB. Inefficient secretion of apoA-V may explain the low circulating concentration of this protein in humans (4). The density distribution of apoB-containing lipoproteins secreted by Hep3B cells transfected with apoA-V was indistinguishable from that of cells transfected with an empty vector, indicating that apoA-V overexpression does not influence apoB-containing lipoprotein particle secretion or lipidation. This result is different from findings reported by Schaap et al. (12), who found that VLDL lipidation, but not particle number, was affected by adenovirus-mediated apoA-V overexpression in mice.

Whereas it is known that apoA-V associates with VLDL in plasma, we used confocal immunofluorescence microscopy to determine whether an association between apoA-V and apoB occurs intracellularly. Immunofluorescence microscopy and immunoprecipitation of cell lysates showed definitively that apoB and apoA-V are located in separate and distinct cell compartments, suggesting that the apoA-V association with VLDL is a postsecretory event. The latter finding, together with our observation that most of the newly secreted apoA-V is found in HDL, suggests that apoA-V may be transported from the cell on HDL and exchange onto VLDL after secretion. Whereas apoB localized to the endoplasmic reticulum and throughout the secretory pathway, apoA-V-GFP localized in a discrete compartment that did not colocalize with apoB. Interestingly, the confocal microscopy data suggest that an intracellular pool of apoA-V exists that escapes the secretory pathway, despite the fact that it is synthesized with a secretory signal peptide. In this regard, apoA-V is similar to the recently described apolipoprotein, apoO, which is partially retained in cells even though it possesses a signal peptide (22). ApoO traffics to lipid droplets within cardiomyocytes. In a similar manner, apoA-V is partially retained in hepatocytes and traffics to cytosolic lipid droplets. Given that TG is the primary lipid component of intracellular lipid droplets, it is conceivable that apoA-V's effects on plasma TG levels are manifest through its interactions with this cellular lipid pool.

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