Published in final edited form as:

Biochem Biophys Res Commun. 2019 April 30; 512(2): 387–391. doi:10.1016/j.bbrc.2019.03.087.

High density lipoprotein promotes nascent apolipoprotein A-V secretion from mRNA transfected cells

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Abstract

Despite its exceptionally low circulating concentration, apolipoprotein (apo) A-V is a potent modulator of plasma triacylglycerol levels. The secretion efficiency of nascent apoA-V was investigated in cultured cells transfected with mRNA. Following transfection of HepG2 cells with wild type apoA-V mRNA, apoA-V protein was detectable in cell lysates by 6 h. At 24 h post transfection, evidence of apoA-V secretion into media was obtained, although most apoA-V was recovered in the cell lysate fraction. By contrast, apoA-I was efficiently secreted into the culture medium. A positive correlation between culture medium fetal bovine serum content and the percentage of apoA-V recovered in conditioned media was observed. When transfected cells were cultured in serum-free media supplemented with increasing amounts of high density lipoprotein, a positive correlation with apoA-V secretion was observed. The data indicate that, following signal sequence cleavage, the bulk of nascent apoA-V remains cell associated. Transit of nascent apoA-V out of cultured cells is enhanced by the availability of extracellular lipid particle acceptors.

Graphical Abstract

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Keywords

apolipoprotein A-V; mRNA transfection; HepG2 cells; HEK293 cells; protein secretion; high density lipoprotein

Introduction

Apolipoprotein (apo) A-V is a member of the class of exchangeable apolipoproteins that functions as a potent modulator of plasma triacylglycerol (TG) homeostasis [1]. ApoA-V is expressed solely in liver and possesses unique properties including insolubility at neutral pH in the absence of lipid [2] and a low plasma concentration (~200 – 400 ng/ml) [3,4]. As a component of circulating plasma lipoproteins, apoA-V functions as an indirect activator of TG lipolysis [5]. At the same time, cell associated apoA-V has been shown to associate with cytosolic lipid droplets (LD) [6]. In addition, apoA-V has been identified as an inducible component of bile [7,8]. To date, the molecular basis for the unusual distribution of apoA-V between these tissue compartments remains poorly understood. The presence of apoA-V in plasma is consistent with the fact that *APOA5* encodes a 23 amino acid secretory signal sequence. Moreover, N-terminal sequencing of plasma apoA-V has verified that its signal sequence is cleaved prior to secretion [9].

Instead of proceeding down the classical secretory pathway to secretion, following co-translational translocation of nascent apoA-V to the ER lumen, some portion of the apoA-V pool has been proposed to undergo “retrograde translocation” back to cytosol [10,11] where it is subject to degradation or, alternatively, LD association. The intracellular pool of apoA-V has been shown to affect intrahepatic TG homeostasis. For example, Schaap et al [12] reported that adenovirus-mediated gene transfer of apoA-V into wild type mice was accompanied by decreased secretion of TG-rich lipoproteins. Shu et al [13] observed that the TG content of livers from *APOA5* transgenic mice fed a chow diet was greater than that of WT mice or *apoA5* (–/–) mice. Likewise, in stably transfected hepatoma cells, oleic acid supplementation-induced TG synthesis led to a reduction in apoA-V secretion and a corresponding increase in cell-associated apoA-V that localized to cytosolic LD [10]. Taken together, the data suggest apoA-V plays a role in intrahepatic TG homeostasis.

To investigate the unusual distribution properties of apoA-V, cultured cells were transfected with mRNAs encoding apoA-V or apoA-I and their respective secretion properties determined. Whereas apoA-I is efficiently secreted, with little protein recovered in cell lysates, under the same conditions apoA-V secretion efficiency is low. Increased apoA-V secretion is observed, however, when cells are cultured in media supplemented with fetal bovine serum (FBS) or exogenous isolated high density lipoprotein (HDL), consistent with the concept that apoA-V secretion is regulated by the availability of extracellular lipoprotein acceptors.

Materials and Methods

Materials and antibodies

Human plasma HDL was obtained from Sigma. Affinity-purified goat anti-human apoA-V was reported earlier [2] and goat anti human apoA-I was from Meridian Life Science. Mouse anti α -tubulin was obtained from GenScript and mouse anti goat IgG-HRP was from Santa Cruz Biotechnology. Goat anti mouse IgG-HRP was purchased from Bio-Rad. mRNAs encoding green fluorescent protein (GFP), WT human apoA-V, WT human apoA-I and a chimera encompassing the signal sequence of apoA-I (residues 1–24) fused to the coding sequence of mature apoA-V (residues 24–343), were from Moderna Inc.

Cell culture

HepG2 cells were purchased from Biosciences Divisional Services at UC Berkeley and cultured in Earl's minimum essential medium (MEM, Caisson Laboratories) supplemented with 10% FBS (Peak Serum), 50 U/mL penicillin, 50 μ g/mL streptomycin (Life Technologies), 1:100 dilution GlutaMax (Life Technologies), 1:100 dilution MEM non-essential amino acids (NEAA, Life Technologies) and 1:100 dilution sodium pyruvate (Life Technologies). Cells were incubated in a humidified incubator with 5% CO₂ at 37 °C. Human embryonic kidney (HEK) 293 cells were a generous gift from Dr. Cynthia Mastick (University of Nevada, Reno). Cells were cultured in Dulbecco's modified Eagle's medium high glucose (DMEM, Caisson Laboratories) supplemented with 10% FBS, 50 U/mL penicillin, 50 μ g/mL streptomycin, 1:100 dilution GlutaMax, 1:100 dilution MEM NEAA. Cells were incubated in a humidified incubator with 5% CO₂ at 37 °C.

mRNA transfections

HepG2 cells were plated at 0.5×10^6 cells/well in 12 well cell culture plates (Sarstedt, Germany). HEK293 cells were plated at 0.2×10^6 cells/well in 12 well plates. On the next day, cells were transfected with 500 ng mRNA using Lipofectamine 3000 (Life Technologies) according to the manufacturer's protocol.

Protein preparation and Western blot

HepG2 or HEK293 cells were grown on 12 well culture plates. Following transfection with mRNA and incubation for the indicated times, conditioned medium was collected and centrifuged at 5,000 x g for 5 min to remove cell debris and the supernatant collected. The cell monolayer was rinsed with PBS, and lysed for 20 min on ice in RIPA buffer containing a protease inhibitor cocktail ProteaseArrest (G Biosciences). Cells were scraped into Eppendorf tubes and the volume adjusted to the volume of medium with PBS. Cells were passed through a 21 gauge needle, centrifuged at 13,000 x g for 20 min and the supernatants collected. Equal volumes of conditioned media and cell lysates samples were loaded into 10% polyacrylamide gels (Bio-Rad) and proteins separated by SDS-PAGE, transferred into a polyvinylidene difluoride membrane (Bio-Rad) and incubated overnight at 4 °C with a given primary antibody, followed by the corresponding secondary HRP-conjugated antibody for 1 h at room temperature. Antibody binding was detected by chemiluminescence using SuperSignal West Pico PLUS Chemiluminescent substrate (Thermo Scientific). α -Tubulin

served as internal standard. Densitometry was applied for protein expression analysis using Image Lab software (Bio-Rad).

Results

Time course studies.

When HepG2 cells were transfected with mRNA encoding GFP, expression of this protein was readily detectable by fluorescence microscopy (data not shown). At the same time, anti apoA-V immunoblot analysis of GFP mRNA transfected cell lysates and conditioned media were negative (Figure 1). Given the low background expression of endogenous apoA-V by HepG2 cells, this cell line represents a suitable model for apoA-V mRNA transfection studies. When WT apoA-V mRNA was transfected into HepG2 cells and the cells cultured in media containing 1.6 % FBS, time course studies revealed the presence of apoA-V in cell lysates at 6 h post transfection, with barely detectable levels present in conditioned media. At 24 h post transfection, a stronger apoA-V band was detected in cell lysates, with comparatively lesser amounts recovered in conditioned media. At 48 h, the apoA-V signal in cell lysates was diminished (likely due to degradation of apoA-V mRNA and protein) while apoA-V levels in conditioned media were similar to that observed at 24 h. Based on this, it was concluded that 24 h post mRNA transfection is optimal for analysis of factors that may affect apoA-V secretion efficiency.

Effect of FBS on apoA-V secretion efficiency.

To further investigate the apparent poor secretion efficiency of apoA-V from cultured hepatoma cells, the effect of culture medium FBS content was evaluated (Figure 2). HepG2 cells were transfected with apoA-V mRNA and the cells cultured in media supplemented with increasing amounts of FBS. At zero or low (0.5 %) FBS levels, the majority of apoA-V protein was recovered in cell lysates. On the other hand, as the FBS content of the culture medium was increased to 2% or 5%, the relative percentage of secreted apoA-V increased, although the bulk of the newly synthesized apoA-V remained intracellular. To confirm that this behavior is unusual and unexpected, control experiments were conducted with a related member of the class of exchangeable apolipoproteins, apoA-I. Given that apoA-I is endogenously expressed in HepG2 cells, transfection with apoA-I mRNA was not required to detect a signal. However, HepG2 cells were also transfected with apoA-I mRNA under conditions identical to those used for apoA-V. Immunoblot results show that, unlike apoA-V, apoA-I is efficiently secreted from HepG2 cells under all culture conditions examined.

Effect of cell type on apolipoprotein secretion efficiency.

Given the large differences in relative secretion efficiency observed between apoA-V and apoA-I, studies were conducted using a different cell line, HEK293. These cells do not express either apoA-I or apoA-V. When HEK293 cells were transfected with apoA-I mRNA and cultured in medium supplemented with 2 % FBS, apoA-I was efficiently secreted (Figure 3). Following transfection of HEK293 cells with apoA-V mRNA and culturing for 24 h in medium supplemented with 2 % FBS, poor secretion efficiency continued to be observed. To examine the possibility that the apoA-V signal sequence may be responsible for this secretion behavior, a chimeric mRNA encoding the apoA-I signal sequence fused to

the mature apoA-V coding sequence was synthesized. Transfection of this mRNA into either HepG2 cells or HEK293 cells led to apoA-V protein production but, in both cells types, the preponderance of apoA-V protein was recovered in the cell lysate fraction. When the effect of culture medium FBS content on the secretion efficiency of WT apoA-V mRNA versus the chimeric mRNA was examined, no differences in secretion efficiency were observed (data not shown), indicating apoA-V's signal sequence is not responsible for the apparent secretion defect.

Effect of HDL on apoA-V secretion efficiency.

The enhancement in apoA-V secretion from cultured HepG2 and HEK293 cells transfected with apoA-V mRNA as a function of culture medium FBS content suggests serum contains an “acceptor” that is capable of binding apoA-V and facilitating its secretion from cells. Based on the known function of apoA-V to associate with plasma lipoproteins and the insolubility of lipid-free apoA-V in neutral pH buffers [2], the hypothesis that HDL serves as acceptor was examined [14]. To test this, following transfection with apoA-V mRNA, HepG2 cells were cultured in media containing 0 % FBS plus indicated amounts of isolated human plasma HDL (Figure 4). The results revealed that, in the absence of HDL, apoA-V secretion efficiency was very low. As the concentration of HDL in the culture medium was increased, however, a corresponding increase in the amount of apoA-V recovered in the culture medium was observed. Control experiments revealed that the HDL used contain no detectable apoA-V when probed by immunoblot.

Discussion

ApoA-V is a unique member of the class of exchangeable apolipoproteins. Given its very low concentration in plasma, it was discovered by a reverse genetics approach rather than classical biochemical methods [1]. Subsequent studies in genetically engineered mice revealed that apoA-V is a potent modulator of plasma TG levels. Moreover, rare mutations that disrupt the *APOA5* gene in humans lead to severe hyperlipidemia and enhanced risk of disease [15]. In addition, a subject carrying a homozygous deletion mutation in the apoA-V signal sequence (Ala6 – Ala13) [16] presented with severe chylomicronemia and no apoA-V in plasma. This report provides evidence indicating a functional signal sequence is required for secretion of apoA-V. In the present study, based on comparative electrophoretic mobility analysis, evidence indicates the signal sequence has been removed from apoA-V recovered in both cell lysates and conditioned media. These data suggest that although nascent apoA-V translocation to the ER and signal sequence cleavage occur normally, an impediment exists that affects the secretion efficiency of this protein.

In studies involving transient transfection of an apoA-V-GFP fusion protein, Shu et al [6] observed that the apoA-V-GFP product does not colocalize with apoB containing lipoproteins in the secretory pathway. This result indicates that apoA-V is not secreted from cells as a component of TG-rich lipoproteins. Given the relative hydrophobicity of apoA-V's amino acid sequence [17] together with its poor solubility as a lipid-free protein [2], it is conceivable that an extracellular “acceptor” is required to promote release of apoA-V from cells. The observation that recombinant wild type apoA-V is fully soluble when complexed

with lipid into reconstituted HDL [18] suggests that, in the absence of an extracellular lipoprotein binding partner, apoA-V secretion is inhibited. On the other hand, no such requirement exists for the related apolipoprotein, apoA-I, which is efficiently secreted from cells even when the cells are cultured in media lacking FBS altogether. Given the known ability of apoA-V to bind HDL in plasma [14], it was hypothesized that the component in FBS that promotes apoA-V secretion from cells transfected with apoA-V mRNA is HDL. Indeed, when serum free media was supplemented with isolated HDL, apoA-V secretion efficiency was strongly enhanced.

It is conceivable that apoA-V traffics onto plasma HDL particles where it resides, poised to associate with post prandial VLDL and chylomicrons through apolipoprotein transfer [19]. In this way, HDL could serve as a reservoir of apoA-V protein that can be accessed in response to elevated plasma TG levels. As TG-rich particles enter the bloodstream, HDL associated apoA-V can transfer to these particles and promote their interaction with glycosylphosphatidylinositol high density lipoprotein binding protein 1, a membrane anchored “receptor” that also serves as a binding site for lipoprotein lipase [5, 19]. In this context, apoA-V serves as an indirect activator of LPL activity, promoting TG hydrolysis and maintenance of plasma TG homeostasis. In the absence of apoA-V, hypertriglyceridemia occurs.

The use of mRNA is new to studies of apolipoprotein secretion. The relative ease of transfection as well as the robust, albeit transient, protein expression observed 24 h post-transfection indicates that mRNA offers a powerful alternative approach to investigate the effect of mutations, polymorphisms and engineered constructs. As shown herein, members of the class of exchangeable apolipoproteins are well suited to mRNA directed expression.

The studies described confirm that nascent apoA-V secretion from cultured cells is inefficient compared to apoA-I. At present, the molecular basis for its unusual behavior is not known although the overall hydrophobicity of the apoA-V amino acid sequence, particularly its central and C-terminal regions [17], may influence protein trafficking in the secretory pathway [11]. It is worth considering that this property may constitute an intrinsic design feature of apoA-V, thereby allowing for retention of apoA-V by cells under certain conditions, such as following partial hepatectomy wherein apoA-V mRNA is strongly upregulated [20]. Moreover, this aspect of apoA-V trafficking may be relevant to its known occurrence as a component of bile [7,8]. The finding that apoA-V levels in bile increase in response to dietary lipid intake suggest a regulated mechanism exists to control apoA-V delivery / entry to bile. Moreover, it is conceivable that inefficient secretion allows for alternative trafficking of nascent apoA-V for purposes that impact whole body TG homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by a grant from the National Institutes of Health (R37 HL64159). The authors thank Colin Fox and Sharon Young for helpful discussions and assistance with figure preparation.

Abbreviations;

apo	apolipoprotein
TG	triacylglycerol
HDL	high density lipoprotein
LD	lipid droplet
FBS	fetal bovine serum
GFP	green fluorescent protein

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Highlights

- Transfection of cells with apoA-V mRNA induced protein production
- Compared to a control apolipoprotein, apoA-V secretion is inefficient
- Culture medium fetal bovine serum content altered apoA-V secretion efficiency
- In serum free medium isolated HDL enhanced apoA-V secretion from HepG2 cells

Figure 1. Effect of apoA-V mRNA transfection on protein expression and secretion from cultured HepG2 cells.

(A) HepG2 cells (0.5×10^6 cells) were transfected with 0.5 μ g GFP mRNA (negative control) or WT ApoA-V mRNA and cultured in media supplemented with 1.6 % FBS. At 6, 24, and 48 h post transfection, conditioned medium and cell lysate fractions were obtained. Equivalent aliquots of these fractions were subjected to SDS-PAGE and anti-apoA-V immunoblot analysis with Anti α -tubulin serving as protein loading control; (B) Densitometric analysis of band intensities. L = cell lysate; M = conditioned medium.

Figure 2. Effect of FBS on the efficiency on nascent protein secretion from HepG2 cells transfected with mRNA.

A) WT apoA-V mRNA (0.5 μ g) was transfected into HepG2 cells (0.5×10^6 cells) and the cells cultured in media supplemented with indicated amounts of FBS. At 24 h post transfection cell lysates and conditioned medium were obtained and equivalent aliquots subjected to SDS-PAGE - anti-apoA-V immunoblot analysis. B) WT apoA-I mRNA (0.5 μ g) was transfected into HepG2 cells (0.5×10^6 cells) and the cells cultured in media supplemented with indicated amounts of FBS. At 24 h post transfection, cell lysates and conditioned medium were obtained and equivalent aliquots subjected to SDS-PAGE - anti-apoA-I immunoblot analysis. Anti α -tubulin served as the protein loading control. L = cell lysate; M = conditioned medium.

Figure 3. Effect of cell type on protein secretion following mRNA transfection.

A) WT apoA-I mRNA, WT apoA-V mRNA or a chimeric mRNA (apoA-I signal sequence – mature apoA-V) were transfected (0.5 μ g RNA) into HepG2 cells (0.5×10^6 cells) and the cells cultured in media supplemented with 1 % FBS. At 24 h post transfection cell lysates and conditioned medium were obtained and equivalent aliquots subjected to SDS-PAGE immunoblot analysis. B) As in panel A except experiments were conducted in HEK293 cells. L = cell lysate; M = conditioned medium.

Figure 4. Effect of media HDL content on apoA-V secretion from HepG2 cells transfected with WT apoA-V mRNA.

A) WT apoA-V mRNA (0.5 μg) was transfected into HepG2 cells (0.5×10^6 cells) and the cells cultured in media supplemented with indicated amounts of HDL. At 24 h post transfection cell lysates and conditioned medium were obtained and equivalent aliquots subjected to SDS-PAGE – anti-apoA-V immunoblot analysis. Anti a-tubulin served as the protein loading control. B) Densitometric analysis of band intensities plotted as the ratio of conditioned media / cell lysate band intensities. L = lysate; M = medium.