The ins (cell) and outs (plasma) of apolipoprotein A-V

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Abstract **Apolipoprotein A-V (apoA-V) has a close interrelationship with plasma triglyceride (TG). Since the discovery of the apoA-V gene in 2001, we have learned that single nucleotide polymorphisms in this gene correlate with altered plasma TG levels in humans, while genetically engineered mice manifest unique TG phenotypes. Studies of recombinant apoA-V protein have revealed that it is composed of two independently folded structural domains. The C-terminal domain possesses high lipid binding affinity, while the N-terminal domain adopts a helix bundle molecular architecture. A sequence element with high positive charge character, between residues 185 and 228, functions in binding of apoA-V to heparan sulfate proteoglycans as well as to members of the low-density lipoprotein receptor family and glycosylphosphatidylinositol high-density lipoprotein binding protein1. These interactions may be related to the capacity of this protein to regulate TG levels. ApoA-V is poorly secreted from transfected cultured hepatoma cell lines and is present in plasma at exceedingly low levels. Studies of apoA-V intracellular trafficking revealed an association with cytosolic lipid droplets. Thus, it is conceivable that apoA-V may also modulate TG metabolism within the cell. Much remains to be learned about this fascinating yet confounding member of the class of exchangeable apolipoproteins.**—Forte, T. M., X. Shu, and R. O. Ryan. **The ins (cell) and outs (plasma) of apolipoprotein A-V.** ***J. Lipid Res.*** **2009.** 50: **S150–S155.**

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Exchangeable apolipoproteins that possess a unique structural feature, the amphipathic α-helix, play an essential role in plasma lipoprotein metabolism. Aberrations in the concentration and/or structure of apolipoproteins are frequently associated with dyslipidemias and increased risk for premature coronary artery disease. A new member of the exchangeable apolipoprotein family was discovered by Pennacchio et al. in 2001 (1). These authors carried out a comparative genomics study in the region of the apolipoprotein (apo) gene cluster (*APOAI/CIII/AIV*) on human chromosome 11q23. Comparison with the corresponding mouse sequence revealed a conserved region ~30 kb proximal to the gene cluster. This new gene, termed *APOAV,* encodes a 366 amino acid protein in humans.

To evaluate the physiological function of apoA-V, Pennacchio et al. (1) created mice that were deficient in murine apoA-V or that overexpressed human apoA-V. Mice expressing the *APOAV* transgene showed a 70% decrease in plasma triglyceride (TG) compared with control littermates, while apoA-V deficient mice had a 4-fold elevation in TG concentration. These early observations suggested that apoA-V has a role in regulating TG metabolism.

While the discovery of Pennacchio et al. was unfolding, van der Vliet et al. (2), using cDNA subtractive hybridization, discovered a gene that is upregulated in regenerating rat liver. The encoded protein, comprising 367 amino acids, was expressed only in liver and was identified as apoA-V. Partial hepatectomy in the rat was followed in the early stages of regeneration by a 5-fold elevation in plasma apoA-V levels, where apoA-V was predominantly associated with HDL. Although the precise role of apoA-V in liver regeneration is unknown, these authors speculated that apoA-V may prevent lipid overload in the regenerating tissue. It is also possible that apoA-V is important in sequestering and/or maintaining intracellular lipid depots that can be utilized by regenerating liver tissue for energy requirements and/or membrane synthesis. Consistent with this, it has been proposed that lipid droplets are a prerequisite for liver regeneration (3, 4). That apoA-V may have a role in cytosolic lipid droplet metabolism is supported by the observation that the protein associates with

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Abbreviations: ADRP, adipocyte differentiation-related protein; apo, apolipoprotein; ER, endoplasmic reticulum; GPIHBP1, glycosylphosphatidylinositol high-density lipoprotein binding protein1; HSPG, heparan sulfate proteoglycan; HTG, hypertriglyceridemia; LDLR, LDL receptor; LRP, LDL receptor-related protein; SNP, single nucleotide polymorphism; TG, triglyceride.

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lipid droplets in hepatoma cell lines transfected with apoA-V (discussed below).

Early studies on apoA-V laid the groundwork for more extensive studies on the role of apoA-V in regulating plasma TG levels and its potential role in regulating intracellular TG metabolism. This review addresses both plasma and intracellular aspects of apoA-V function.

APOA-V AND ATHEROSCLEROSIS

Because TG is a known independent risk factor for the development of cardiovascular disease and since apoA-V was identified as a modulator of TG levels, a number of studies have sought to establish a genetic link between apoA-V and TG. Pennacchio et al. (1, 5) were the first to note that several single nucleotide polymorphisms (SNPs) in the apoA-V gene, particularly –1131T>C and c.56C>G (S19W), were associated with elevated TG levels. Similar results were obtained by others (6–9). Several approaches have been used to determine whether the rare S19W SNP is functional, as it correlates strongly with elevated TG. Based on the fact that this coding SNP occurs in the signal peptide region of the protein, Talmud et al. (10) hypothesized that its secretion efficiency may be affected. Consistent with molecular modeling studies that predict reduced translocation of this variant across the endoplasmic reticulum (ER), studies in transfected HepG2 cells demonstrated that the S19W variant was indeed poorly secreted (50% reduction). The role of the S19W variant was also investigated by precise insertion of a single-copy 19W APOA5 haplotype into the mouse genome (11). This elegant set of studies revealed that the S19W polymorphism is functional because it resulted in a significant decrease in plasma apoA-V levels. Since there is a strong correlation between hypertriglyceridemia (HTG) and atherogenesis, Talmud et al. (6) examined atherogenic lesion progression in subjects with the S19W polymorphism. The data showed a trend toward increased atherogenesis in the vessel lumen, although it is difficult to draw firm conclusions from these data. Future studies with atherogenic animal models, where variants can be expressed, could provide clues in this area. Interestingly, a recent study using the dyslipidemic apoE2-knockin mouse model demonstrated that expression of human apoA-V not only reduced plasma TG levels but also reduced atherosclerotic lesion formation in apoA-V transgenic mice compared with controls (12). This is perhaps the strongest evidence that apoA-V may have antiatherogenic potential; however, apoA-V metabolism is complex, and one needs to take into consideration the contribution of other lipid-regulating factors, such as apoCIII, apoCII, and LPL, as well as contributions from environmental factors, such as diet, obesity, diabetes, etc.

PLASMA CONCENTRATIONS OF APOA-V

In humans, the plasma concentration of apoA-V, compared with other apolipoproteins, is exceptionally low. Estimates range from 114 to 258 ng/ml in normolipemic subjects (13–17). This corresponds to a 1,000-fold lower concentration than apoB and a 10,000-fold lower concentration than apoA-I on a molar basis. Early studies (1) in *APOAV* transgenic and gene knockout mice indicated an inverse relationship between apoA-V and plasma TG levels (i.e., in the absence of apoA-V, plasma TG levels were highly elevated, whereas overexpression of human apoA-V lowered TG compared with controls). Adenovirus-mediated gene transfer of apoA-V in mice also resulted in a dose-dependent lowering of TG (18, 19). Various human population studies relating plasma apoA-V and TG do not wholly support the concept of an inverse relationship between these two variables. On the contrary, several studies with HTG and/or diabetic subjects revealed that apoA-V increased as TG increased (15–17, 20). Also, a positive correlation has also been observed in normolipemic controls (16, 17). Human apoA-V transgenic mice with normal TG levels are similar to their human counterparts in that there is a significant positive correlation between apoA-V and plasma TG concentrations (21), thus establishing that the positive correlation is not confined to human subjects.

The interactions between apoA-V and TG are complex, and many issues need to be resolved (e.g., what regulates the positive relationship between plasma apoA-V and TG in normotriglyceridemic humans and mice?). Is it dependent on the rate of secretion of apoA-V? If so, are TG secretion and apoA-V secretion coupled? It has yet to be unequivocally demonstrated that newly formed apoA-V is secreted into the plasma on a lipoprotein carrier and whether the carrier is VLDL or HDL. Studies on human apoA-V overexpressed in hepatoma cells suggest that apoA-V is not associated with apoB intracellularly but does appear on VLDL and HDL in the medium (22). HDL is the major carrier of apoA-V in human (23) and mouse plasma (21). It is unclear, however, whether this distribution reflects direct secretion on HDL or if HDL is serving as a postlipolytic reservoir for the protein.

UNIQUE ASPECTS OF APOA-V STRUCTURE

Mature apoA-V (343 amino acids), which lacks the 23 amino acid signal peptide, is a highly hydrophobic protein that possesses considerable α-helix secondary structure and is largely insoluble as a lipid-free protein in aqueous solution (24). The apoA-V amino acid sequence reveals the presence of a unique tetraproline sequence element near the C terminus of the protein. The hypothesis that this sequence element demarcates a boundary between independently folded structural domains in apoA-V has been examined (25). To evaluate the role of this C-terminal segment, a truncated apoA-V comprising amino acids 1 to 292 was generated. Far-ultraviolet circular dichroism spectra of full-length apoA-V and apoA-V(1-292) were similar, with ~50% α-helix content. In guanidine HCl denaturation experiments, both full-length and C-terminal truncated apoA-V yielded biphasic profiles consistent with the presence of two structural domains. ApoA-V(1-292) dis-

played an attenuated ability to solubilize dimyristoylphosphatidylcholine vesicles compared with full-length apoA-V, whereas a peptide corresponding to the deleted C-terminal segment displayed markedly enhanced lipid solubilization kinetics (25). The data support the concept that the C-terminal region is not required for apoA-V to adopt a folded protein structure yet functions to modulate apoA-V lipid binding activity. As such, the extreme C terminus of apoA-V may be relevant to the mechanism whereby apoA-V influences plasma TG levels.

Computer-assisted sequence analysis and limited proteolysis studies identified a 146 amino acid N-terminal fragment as a candidate for one of the apoA-V domains. Unlike the full-length protein, recombinant apoA-V(1-146) was soluble in neutral-pH buffer in the absence of lipid (26). Guanidine HCl denaturation experiments at pH 3.0 yielded a one-step native-to-unfolded transition that corresponds directly with the more stable component of the two-stage denaturation profile exhibited by full-length apoA-V. Taken together, the data suggest that the N-terminal 146 residues of human apoA-V adopt a helix bundle molecular architecture in the absence of lipid and thus likely exist as an independently folded structural domain within the context of the intact protein. Thus, it appears that apoA-V may be organized in a manner that is similar to apoE and apoA-I, being composed of a lipid-surface-seeking C-terminal domain and an α-helix bundle-forming N-terminal domain. The biological relevance of this domain structure has yet to be determined.

EXTRACELLULAR ROLE OF APOA-V IN TG METABOLISM

Between residues Glu185 and Asp228, mature apoA-V possesses a cluster of positively charged residues. Based on precedent with other exchangeable apolipoproteins (e.g., apoE), the hypothesis that this positively charged cluster interacts with heparin was tested. Data obtained revealed that apoA-V bound to a heparin affinity column and a heparin-coated surface plasmon resonance chip (27). At the same time, Merkel et al. (28) reported that apoA-V accelerates plasma hydrolysis of TG-rich lipoproteins by facilitating interaction with heparan sulfate proteoglycan (HSPG)-bound LPL but not with free LPL. Thus, it is conceivable that apoA-V functions to modulate plasma TG levels by directing substrate lipoproteins to target cell surface HSPG, thereby presenting TG substrate to LPL (**Fig. 1**).

In an effort to define the heparin binding domain in apoA-V, site-directed mutagenesis studies were performed (29). When positively charged residues between residues 210 and 220 were mutated to either negatively charged amino acids or to uncharged polar amino acids, a decrease

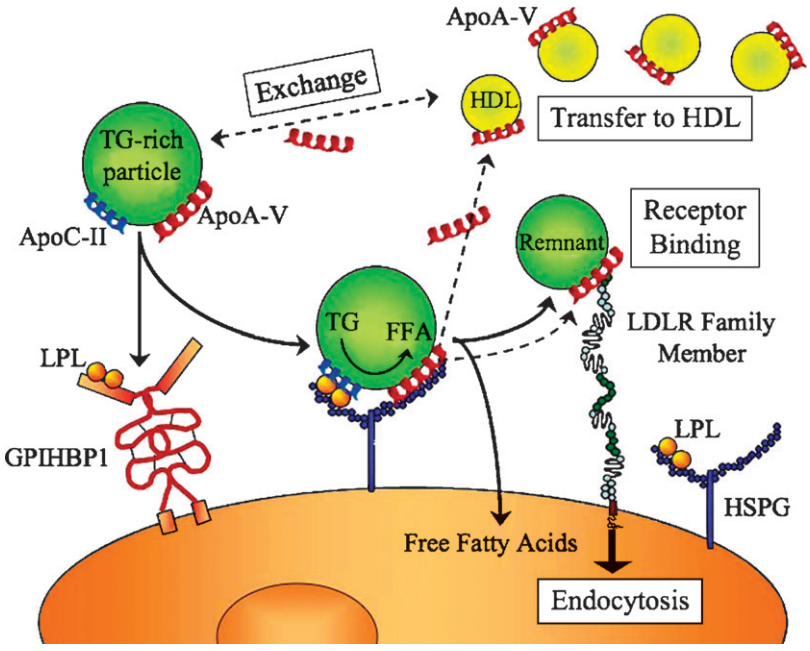

Fig. 1. Postulated extracellular effects of apoA-V on TG-rich lipoprotein metabolism. ApoA-V interaction with HSPGs can facilitate apoC-II activation of LPL, resulting in accelerated TG hydrolysis. Catabolism of TG-rich lipoproteins [VLDL and chylomicrons (CM)] to a remnant particle may result in apoA-V dissociation from TG-rich particles and transfer to plasma HDL, where it remains poised to exchange onto nascent plasma TG-rich lipoproteins. ApoA-V binding to GPIHBP1 could facilitate hydrolysis of TG in CM. It has been proposed that the GPIHBP1 protein forms a homodimer whose negatively charged domains bind LPL and apoA-V, leading to efficient lipolytic processing of CM (45). ApoA-V interaction with LDLR family members could facilitate endocytosis of remnant lipoproteins. Solid arrows represent the path of lipoprotein particles; dashed arrows represent the path of apoA-V. Adapted from Wong and Ryan (46) with permission.

in heparin binding activity was observed. Based on this, one can conclude that apoA-V interacts with HSPGs via the positively charged sequence element in the central region of the protein.

A unique SNP (c.553G>T) found in subjects of Asian origin substitutes a cysteine for glycine at residue 162 of the mature protein (30–32). Carriers of the T allele had TG concentrations twice that of normolipidemic subjects, while TT homozygous subjects had extremely elevated TG (mean of 2,292 mg/dl). The T allele was also associated with a decrease in LPL activity. Given the fact that individuals harboring this SNP manifest elevated plasma TG levels, it is conceivable that intramolecular disulfide bond formation between Cys204, the only naturally occurring cysteine in apoA-V, and Cys162 may disrupt the conformation of the positively charged segment of the protein and interfere with its ability to interact with HSPGs. Formation of an intramolecular disulfide bond has yet to be demonstrated and awaits future experimentation.

The presence of a positively charged sequence element in apoA-V suggests that interaction with members of the LDL receptor (LDLR) family may also contribute to plasma TG lowering effects of apoA-V. Indeed, studies of apoA-V-deficient mice revealed that elevated plasma TG may be the result of a lower affinity of apoA-V-deficient VLDL for the LDLR (33). Such an effect is consistent with the concept that apoA-V itself may be a ligand for members of the LDLR family (Fig. 1). Recent studies demonstrated the ability of apoA-V to interact with members of the LDLR family, including the LDL receptor-related protein (LRP) and the mosaic type I receptor SorLA (29). Preincubation with heparin decreased apoA-V binding, indicating that overlap exists between the recognition sites for these receptors and for heparin. A double mutant, apoA-V (Arg210Glu/Lys211Gln), showed decreased binding to heparin and decreased ability to bind LRP. Independent confirmation that apoA-V can serve as a ligand for members of the LDLR family was reported by Dichlberger et al. (34). These authors showed that apoA-V serves as a ligand for LDLR family members in the laying hen, confirming results with human apoA-V.

Experiments using surface plasmon resonance showed specific binding of apoA-V to another member of the LDLR family, sortilin (35). Unlike LRP and SorLA, however, sortilin lacks LDL-A repeats but contains the Vps10p domain. Binding of apoA-V to sortilin was competed by neurotensin, a ligand that binds specifically to the Vps10p domain. Binding experiments conducted in transfected human embryonic kidney cells revealed enhanced binding of apoA-V to SorLA/LR11- and sortilin-expressing cells compared with control cells. Internalization experiments demonstrated that labeled apoA-V was rapidly internalized, colocalized with receptors in early endosomes, and followed the receptors through endosomes to the trans-Golgi network. It can be concluded that apoA-V binds to receptors possessing LDL-A repeats or Vps10p domains and that apoA-V is internalized into cells via these receptors. While this could be a mechanism by which apoA-V modulates TG metabolism in vivo, it needs to be explored further.

Beigneux et al. (36) provided additional evidence for the concept that apoA-V can modulate plasma TG levels by promoting cell surface interactions. These authors reported that apoA-V serves as a ligand for a novel endothelial cell surface protein, glycosylphosphatidylinositol high-density lipoprotein binding protein1 (GPIHBP1). *Gpihbp1*-deficient mice exhibit a striking accumulation of chylomicrons, even on a low-fat diet, resulting in milky plasma with TG levels as high as 5,000 mg/dl. On the basis of these studies, it was concluded that GPIHBP1 plays a critical role in the lipolytic processing of chylomicrons. GPIHBP1 is located on the luminal face of the capillary endothelium, and its expression in cultured cells confers an ability to bind both LPL and TG-rich particles, such as chylomicrons (Fig. 1). In studies with *GPIHBP1* transfected carbohydrate cells, specific binding to apoA-V-containing lipid particles was observed. Mutation of the positively charged heparin binding domain within apoA-V abolished its ability to bind to GPIHBP1 (37). These studies provide further evidence that apoA-V may play a role in chylomicron TG hydrolysis.

INTRACELLULAR ROLE OF APOA-V IN TG METABOLISM

Whereas the studies discussed above provide convincing evidence that apoA-V has a role in regulating extracellular TG concentrations, its intracellular role in TG metabolism is more ambiguous. Studies by Schaap et al. (38) with adenovirus-mediated gene transfer of *apoa5* into C57Bl/6 mice indicated that expression of apoA-V downregulated hepatic production of VLDL TG, resulting in the formation of smaller VLDL particles, while particle numbers were not affected. These authors suggested that intracellular apoA-V impairs apoB lipidation. Using apoA-V transgenic mice, several laboratories (28, 39) demonstrated that overexpression of human apoA-V did not significantly alter VLDL TG production rates compared with controls. Similar results were obtained with apoA-V knockout mice compared with control littermates (33). Multicompartmental modeling kinetics with human subjects also suggested that there is no correlation between apoA-V levels and VLDL production (40). Although it is not immediately evident why results with mice are discrepant, the method of expressing the protein (transgene versus adenovirus transfer) may play a role in the outcomes of the experiments.

Our laboratory has shown that, although apoA-V did not associate with apoB in Hep3B cell lysates, it was associated with VLDL isolated from the culture medium, suggesting that apoA-V binding to VLDL is a postsecretory event (22). The density distribution and amount of apoB containing lipoproteins secreted by Hep3B cells transfected with human apoA-V was indistinguishable from that of nontransfected cells, indicating that apoA-V expression did not influence apoB-containing lipoprotein secretion or lipidation. Since apoA-V has a role in VLDL TG metabolism, the expectation was that apoA-V associates with apoB intracellularly and is secreted with nascent VLDL. Studies with hepatoma cell lines indicate that this is not the case; thus,

one might ask, "how is apoA-V transported into the medium?" One cannot rule out that apoA-V may be transported on a nascent HDL or alternatively that the protein associates with the hepatocyte cell membrane similar to apoE and lipoprotein association is an extracellular event. These mechanisms remain to be explored.

APOA-V AND INTRACELLULAR LIPID DROPLETS

To determine where apoA-V was localized in liver cells, our laboratory transfected the Hep3B cell line with a human apoA-V-green fluorescent protein fusion protein and its intracellular distribution was visualized by confocal microscopy. Whereas apoA-V did not colocalize with apoB, surprisingly, it was found associated with cytosolic lipid droplets (22). Additional studies with transfected McA-RH7777 cells revealed that apoA-V colocalizes with adipocyte differentiation-related protein (ADRP), a specific marker for lipid droplets (41), confirming its lipid droplet association. This trafficking path was totally unexpected because apoA-V possesses a signal sequence that destines it for secretion. However, trafficking of apoA-V to intracellular lipid droplets is not unique to this apolipoprotein, for it was recently demonstrated that apoO in H9c2 cardiomyocytes also traffics to lipid droplets even though it too possesses a signal peptide (42).

In hepatocytes, the cytosolic lipid droplet constitutes a dynamic organelle that is composed primarily of TG surrounded by a phospholipid monolayer and associated proteins. It is tempting to speculate that apoA-V may function like many of the known lipid-droplet-associated proteins, including perilipin, ADRP, and TIP47 (collectively known as members of "PAT" family proteins) to help stabilize/modulate these lipid depots. X-ray crystallography of the C-terminal domain of TIP47 revealed that this lipid-droplet-associated protein has a helix bundle motif similar to the N-terminal helix bundle of exchangeable apolipoproteins, such as apoE (43). It has been postulated that the helix bundle can "open" upon lipid interactions, thereby exposing a hydrophobic domain that can bind to a lipid surface. The N-terminal region of apoA-V possesses such a helix bundle motif that could potentially bind the surface of cytosolic lipid droplets. Additionally, apoA-V has a highly hydrophobic C-terminal domain shown to avidly bind lipid (25). The presence of this hydrophobic region is likely important in apoA-V's interaction with lipid droplets. Indeed, studies with the apoA-V(1-146) variant revealed that this truncated protein does not home to lipid droplets (41).

Newly synthesized proteins expressing the signal peptide sequence normally transit the ER and the secretory pathway for ultimate secretion. Thus, it is surprising that apoA-V in cultured hepatoma cell lines homes to lipid droplets. One possible explanation is that because of its high expression in transfected Hep3B and McA-RH7777 cell lines, the protein, by default, traffics to lipid droplets where its interaction with lipid may protect it from intracellular degradation. On the other hand, apoA-V's association with lipid droplets may represent a normal intracellular path for the protein.

Factors that regulate lipid droplet homing of apoA-V are unknown but may be related to its unique molecular features. Although speculative, the signal peptide may direct newly synthesized apoA-V to the ER compartment, which is also the site of lipid droplet genesis. Nascent lipid droplet assembly occurs within the membrane of the ER, and, as it accrues sufficient lipid, a droplet is formed that ultimately buds into the cytosol and later pinches off as a discrete organelle (44). Because of its high lipid affinity, apoA-V, while transiting the ER, may bind to the nascent lipid droplet and subsequently be retained on the mature cytosolic droplet. However, the functional role of apoA-V on the surface of lipid droplets is unknown. It may serve as a scaffolding protein to help maintain the integrity of the droplet or it may potentially have a role in regulating TG hydrolysis in the lipid droplet. These are areas to be explored in the future.

CONCLUSIONS

Evidence is compelling that apoA-V has a physiological role in the extracellular metabolism of TG. Studies using an array of approaches indicate that apoA-V is an important modulator of plasma LPL activity by increasing the efficiency of VLDL TG hydrolysis. Likewise, by stimulating LPL activity, apoA-V may contribute to hepatic clearance of VLDL remnants. On the other hand, the intracellular role of apoA-V on VLDL TG metabolism is equivocal, and its role in inhibiting VLDL TG production remains controversial. The intracellular localization of apoA-V with cytosolic lipid droplets is unique and could potentially provide a new perspective on apoA-V's role in TG homeostasis.

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