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Influence of apolipoprotein A-V on hepatocyte lipid droplet formation

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ABSTRACT

Apolipoprotein A-V (apoA-V) is postulated to modulate intra-hepatic triglyceride (TG) trafficking. Stably transfected McA-RH7777 hepatocarcinoma cells expressing human apoA-V displayed enhanced neutral lipid staining while conditioned media from these cells had 40 ± 8% less TG than cells transfected with a control vector. To obtain homogeneous cell lines expressing different amounts of apoA-V, a strategy of clonal selection was pursued. Immunoblot analysis of two distinct apoA-V stable cell lines yielded one that expresses low amounts of apoA-V and another that expresses higher amounts. Confocal fluorescence microscopy of control cells and cells expressing low levels of apoA-V had similar numbers of lipid droplets while cells expressing higher amounts of apoA-V had twice as many lipid droplets, on average. Thus, apoA-V expression promotes lipid droplet accumulation in these cells.

1. Introduction

The strong positive correlation between hypertriglyceridemia (HTG) and susceptibility to cardiovascular disease has fostered efforts to identify and characterize factors that regulate triglyceride (TG) metabolism. Genome-wide association studies have identified major contributors to HTG, including apolipoprotein (apo) A-V [1,2]. The impact of apoA-V on plasma TG was vividly illustrated in genetically engineered mice [3]. In *APOA5* transgenic mice, plasma TG was 3-fold lower than control littermates while *apoa5* (−/−) mice displayed a 4-fold increase. Moreover, human population studies reveal a strong association between common *APOA5* single nucleotide polymorphisms and plasma TG [4]. Given these associations, defining the molecular basis of apoA-V function is key to translating knowledge of this protein into new approaches for diagnosis, prevention and/or treatment of HTG and related pathological conditions. Practically speaking, plasma TG levels may be influenced by extracellular events (e.g. the rate of lipolysis / clearance of TG-rich lipoproteins) or intracellular events related to production / secretion of TG-rich lipoproteins by liver.

The liver is the only tissue that expresses apoA-V [3,5]. Interestingly, following partial hepatectomy in rats, apoA-V mRNA increases 3.5-fold [5]. Although the function of apoA-V in this physiological setting is unknown, it is likely that, during tissue regeneration, liver cells are programmed to conserve lipid for membrane biogenesis as opposed to its secretion on lipoprotein particles. In another study, it was noted that apoA-V possesses considerable hydrophobic character, and this feature may be related to its poor secretion efficiency [6]. Following adenovirus-mediated overexpression in mice [7,8] or transient overexpression in Hep3B cells [9], apoA-V did not associate with apoB-100 containing lipoprotein particles. On the other hand, Shu et al. [10,11] found that ~50% of newly synthesized apoA-V is retained in the cell in association with cytosolic lipid droplets Walther and Farese [12].

Pamir et al. [13] reported that *APOA5* transgenic mice fed a diet high in fat and sucrose secrete lower amounts of TG. Likewise, apoA-V expression in stably transfected McA-RH7777 cells resulted in decreased TG secretion in a dose dependent manner, generating a VLDL particle population that was ~40% smaller in volume [14]. In the present study stably transfected McA-RH7777 cells expressing human apoA-V were generated to address whether apoA-V expression drives formation of intracellular lipid droplets. The results provide evidence that apoA-V redirects intracellular TG toward lipid droplet assembly at the expense of TG-rich lipoprotein secretion.

2. Material and methods

2.1. Cell culture

McA-RH7777 cells were purchased from American Type Culture Collection (ATCC); growth medium consisted of DMEM containing 10% FBS (Phenix Research), 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma). Stable cell lines were maintained in DMEM growth medium with added 0.25 μg/ml amphotericin B (Sigma) and 100 μg/ml G418 (Invitrogen). Cells were grown in T75 flasks at 37 °C in an atmosphere containing 5% CO_2. The medium was changed every other day and cells were passaged every 4–5 days.

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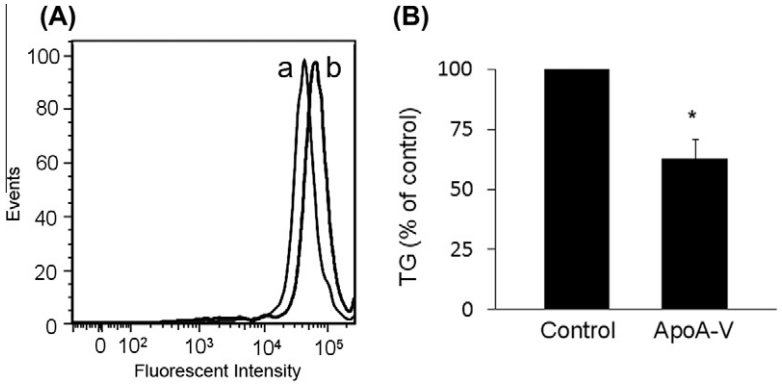

Fig. 1. The effect of apoA-V expression on TG fate in McA-RH7777 cells. (Panel A) Stably transfected McA-RH7777 cells were detached from plates, fixed with 4% paraformaldehyde, washed with PBS and stained with Nile Red prior to flow cytometry. Curve (a) depicts the fluorescence intensity of cells transfected with pcDNA 3.1 control vector while curve (b) shows intensity of cells transfected with human apoA-V pcDNA 3.1. (Panel B) Cells were incubated with serum-free medium 24 h prior to harvesting conditioned media. The conditioned media were concentrated, dialyzed against PBS and TG content measured. Values are presented as percentage of TG secreted by control cells expressed as mean ± S.E.M. ($n = 3$). Student t-test was used to examine statistical difference between apoA-V expressing cells and control cells; $^{*}p \leqslant 0.05$.

2.2. Stable transfection

The full-length human apoA-V sequence was subcloned into pcDNA 3.1 and the resulting construct was verified by sequencing. McA-RH7777 cells in 60 mm dishes were transfected at about 30% confluence with 8 μg of apoA-V pcDNA 3.1 or control pcDNA 3.1 plasmid, using Lipofectamine 2000 (Invitrogen). Twenty-four hours post-transfection, cells were subjected to selection with DMEM-10% FBS supplemented with 200 μg/ml G418. Selection medium was replaced every other day for 14 days. Individual clones were isolated, expanded, and maintained in 100 μg/ml G418.

2.3. Lipid analysis

Stably transfected McA-RH7777 cells were grown in T75 flasks until 30% confluent. Cells were incubated with serum-free medium 24 h prior to harvesting conditioned medium. Following collection, conditioned media were concentrated, dialyzed against phosphate buffered saline (PBS) and TG measured by L-Type Triglyceride M assay (Wako). Measured TG concentrations were normalized to total cell number. Values reported are percentages of the TG level secreted by control cells transfected with empty vector. Student's t-test was used to examine statistical differences between cells expressing human apoA-V and control cells; p-value $\leqslant 0.05$ is considered significant.

2.4. Flow cytometry

Stably transfected McA-RH7777 cells were detached from plates with cell dissociation buffer (Invitrogen), washed with PBS, fixed with 4% paraformaldehyde (Sigma) in PBS for 20 min on ice and neutral lipids stained with Nile Red (Sigma) according to Greenspan et al. [15] prior to flow cytometry on a BD LSRFortessa instrument. FlowJo software was used for data processing.

2.5. Confocal fluorescence microscopy

Stably transfected McA-RH7777 cells were plated on coverslips (Zeiss). Twenty-four hours post plating, cells were fixed with 4% paraformaldehyde in PBS for 20 min, washed three times with PBS and incubated with Nile Red for 20 min. Cell nuclei were counterstained with Hoechst (Invitrogen) for 20 min and washed three times with PBS. Cells were mounted with vectashield mounting medium (Vector) and viewed with a Zeiss LSM710 Confocal Microscope with a 63× oil objective. Photoshop software was used for lipid droplet analysis. Student's t-test was performed with a p-value $\leqslant 0.05$ considered significant.

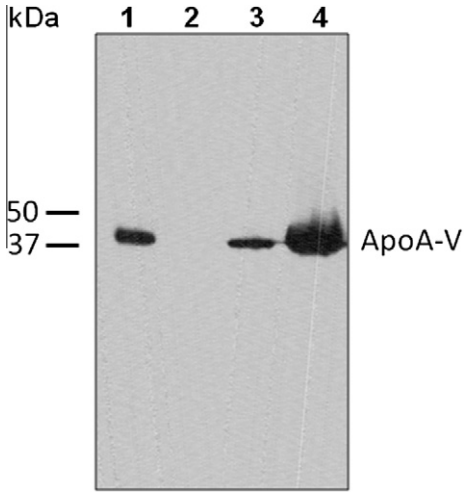

Fig. 2. Immunoblot analysis of clonal lines of stably transfected McA-RH7777. Cell lysates were separated by SDS–PAGE, transferred to a polyvinylidene difluoride membrane and probed with antibodies against apoA-V. (Lane 1) recombinant apoA-V standard (90 ng); (Lane 2) cells transfected with empty vector; (Lane 3) apoA-V pcDNA 3.1 transfected Clone A and (Lane 4) apoA-V pcDNA 3.1 transfected Clone B. Equal amounts of cell protein were loaded in lanes 2–4.

2.6. Immunoblot analysis

Cells were detached from plates with cell dissociation buffer (Invitrogen), washed with PBS, lysed with cold CytoBuster protein extraction reagent (Novagen) lysis buffer plus protease inhibitor cocktail (Sigma) on ice. Total protein amounts in cell lysates were determined by BCA assay (Pierce). Cell lysate contents were separated by electrophoresis on Bio-Rad precast 4–20% acrylamide gradient gels and transferred to 0.2 μm polyvinylidene difluoride membranes using the Trans-Blot Turbo Transfer System (Bio-Rad). The blot was blocked with 20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20 (TBST), containing 5% non-fat milk, and then probed with polyclonal goat anti-human apoA-V solution at 4 °C overnight. After washing with TBST, the membrane was incubated with horseradish peroxidase-conjugated bovine anti-goat IgG secondary antibody (Santa Cruz) for 1 h at room