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Research Paper

ATP-Binding Cassette Transporter A1 Deficiency in Human Induced Pluripotent Stem Cell-Derived Hepatocytes Abrogates HDL Biogenesis and Enhances Triglyceride Secretion

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ABSTRACT

Despite the recognized role of the ATP-binding Cassette Transporter A1 (ABCA1) in high-density lipoprotein (HDL) metabolism, our understanding of ABCA1 deficiency in human hepatocytes is limited. To define the functional effects of human hepatocyte ABCA1 deficiency, we generated induced pluripotent stem cell (iPSC)-derived hepatocyte-like cells (HLCs) from Tangier disease (TD) and matched control subjects. Control HLCs exhibited robust cholesterol efflux to apolipoprotein A-I (apoA-I) and formed nascent HDL particles. ABCA1-deficient HLCs failed to mediate lipid efflux or nascent HDL formation, but had elevated triglyceride (TG) secretion. Global transcriptome analysis revealed significantly increased *ANGPTL3* expression in ABCA1-deficient HLCs. Angiopoietin-related protein 3 (ANGPTL3) was enriched in plasma of TD relative to control subjects. These results highlight the required role of ABCA1 in cholesterol efflux and nascent HDL formation by hepatocytes. Furthermore, our results suggest that hepatic ABCA1 deficiency results in increased hepatic TG and ANGPTL3 secretion, potentially underlying the elevated plasma TG levels in TD patients.

1. Introduction

Tangier disease (TD), a rare autosomal recessive disorder, is characterized by the near absence of plasma high-density lipoprotein (HDL), elevated triglyceride (TG) levels and sterol deposition in tissue macrophages (Clifton-Bligh et al., 1972). TD is caused by mutations in the *ABCA1* gene (Oram and Heinecke, 2005), which encodes the integral membrane protein ATP-binding cassette transporter A1 (ABCA1). ABCA1 facilitates transport of cellular free cholesterol (FC) and phospholipids (PL) to lipid-poor apolipoprotein A-I (apoA-I). Studies with murine models have demonstrated the pivotal role of hepatic ABCA1 in promoting nascent HDL formation and maintaining normal plasma HDL levels (Bi et al., 2013; Timmins et al., 2005). However, primary hepatocytes from TD patients have never been investigated and thus the intrinsic functional effects of the loss of human hepatocyte ABCA1 have yet to be established.

Reprogramming differentiated somatic cells to induced pluripotent stem cells (iPSCs) enables re-differentiation to a wide array of cell types (Yamanaka, 2012) and is of considerable value for disease modeling, functional genomics, drug discovery, and regenerative medicine. The Next Generation Genetic Association Studies consortium was developed to exploit the power of iPSC-derived cells for gaining insight into the functional implications of human genetic variation, and has generated iPSCs from thousands of patients with defined genetic variations

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(http://www.wicell.org/home/stem-cell-lines/collections/collections.cmsx). The liver plays a crucial role in many physiological processes, including lipid and lipoprotein metabolism and the differentiation of human iPSCs to hepatocyte-like cells (HLCs) provides a model system to study hepatocyte-specific functions of human disease and gain mechanistic insights.

In the current study, iPSCs from TD and matched control subjects were generated and differentiated into HLCs. The TD HLCs were shown to have severely impaired cholesterol efflux and nascent HDL formation, as well as increased TG secretion. Gene expression analysis of TD and control HLCs revealed an increase in *ANGPTL3* expression, confirmed by assay of this protein in the media and plasma. These results illustrate the utility of iPSC-derived HLCs in disease modeling, highlight the importance of human hepatic ABCA1 in both HDL and TG metabolism, and show that ABCA1 deficiency results in upregulation of *ANGPTL3*, which is known to influence HDL and TG metabolism.

2. Materials and Methods

All human protocols for the study were approved by the University of Pennsylvania Human Subjects Research Institutional Review Board. The participants provided their informed written consent for the study. The study was conducted according to standards indicated by the Declaration of Helsinki.

2.1. Flow Cytometry Analysis of Pluripotency Markers

iPSCs were detached and dissociated into single cells with Accutase Enzyme Cell Detachment Medium (Innovative Cell Technologies) and gentle pipetting. Cells were then stained with Alexa Fluor 647-SSEA4 (Biolegend 330408), PE-Tra-1-60 (BD Bioscience 560193) or corresponding isotype controls (Biolegend 401321 and BD Bioscience 555584) for cell surface expression analysis by Flow Cytometry using BD FACSCalibur (BD Biosciences, San Jose, CA).

2.2. Karyotypic Analysis of iPSCs

iPSCs were cultured in T25 flasks and live cultures were sent to WiCell Cytogenetics Lab (Madison, WI) for cytogenetic analysis using G-banded Karyotyping. An average of 20 cells per cell line was analyzed for chromosome integrity.

2.3. Generation of Subject-Specific iPSCs

Subject-specific PBMC-derived iPSCs were generated using Sendai viral vectors by the iPSC Core Facility at the University of Pennsylvania as previously described (Yang et al., 2012; Zhang et al., 2015). These cells lines have been deposited at WiCell Research Institute.

2.4. Differentiation of iPSCs to Hepatocytes

HLCs were generated from iPSCs using standard protocols as reported (Mallanna and Duncan, 2013; Si-Tayeb et al., 2010).

2.5. Lipid Efflux

Lipid efflux assays were performed as previously described (Lyssenko et al., 2013). Twenty days after differentiation, HLCs were radiolabeled, with 0.12 μCi/ml ^{14}C-cholesterol and 1.3 μCi/ml ^{3}H-choline or with 0.12 μCi/ml ^{14}C-cholesterol only (PerkinElmer, Waltham, MA, USA) in the presence of T0901317 (10 μM, Sigma-Aldrich T2320) for 24 h. Cells were then washed twice and incubated in fresh medium with or without exogenous human apoA-I (20 μg/ml) for 6 h. At the end of incubation, cell medium was collected and filtered through a 96-well filter plate (EMD Millipore) to remove cell debris. ApoB-containing lipoprotein was precipitated using phosphotungstate. Remaining HDL-containing medium underwent lipid extraction by Bligh-Dyer method. Cellular lipids were extracted with hexane-isopropanol (3:2, *v*/v) and the solvent was then evaporated for scintillation counting. The percentage of cholesterol export was calculated by dividing the ^{14}C counts in the medium by the sum of counts in the medium and cells and multiplying by 100.

2.6. Nascent HDL formation

Nascent HDL formation and gel-filtration chromatography analysis of nascent HDL were performed as previously described (Lyssenko et al., 2013). Briefly, HLCs were dual radiolabeled with 1.3 μCi/ml ^{3}H-choline and 0.12 μCi/ml ^{14}C-cholesterol (PerkinElmer, Waltham, MA, USA) in the presence of 10 μM T0901317 (Sigma-Aldrich T2320) for 24 h. After two washes, efflux medium with or without human apoAI (20 μg/ml) were added to cells for a 6-hour incubation. Cell medium was collected, filtered through a 0.45 μm PVDF membrane filter unit (EMD Millipore), and concentrated using Amicon Ultracel-10 K centrifugal filters (EMD Millipore). A 1-ml aliquot of the concentrated cell medium was resolved into 1-ml fractions on a calibrated HiLoad 16/60 Superdex 200 gel-filtration column (GE Healthcare, Mickleton, NJ, USA). ^{3}H and ^{14}C counts in each fraction were determined by scintillation counting. Results were presented as counts per fraction for regions containing HDLs (fraction 45–90).

2.7. TG Secretion

HLCs were preloaded with oleic acid (1.5 mM) in the presence of ^{3}H-glycerol (5 μCi/ml) for 6 h. Culture media samples were collected for low speed spin to remove cell debris. Supernatant from the spin was harvested. An aliquot of media was used for lipid extraction by Bligh-Dyer method while another portion was used for albumin quantification using ELISA (BETHYL Laboratories E88–129). Cellular lipids were extracted with isopropanol, after which 0.1 N NaOH was added to collect cell lysates. Lipid extracts from medium and cells were used for thin layer chromatography and scintillation counting. Lowry protein assay was performed to measure cellular protein contents. ^{3}H-TG counts in cells and medium were normalized to medium albumin mass to determine TG secretion (Chung et al., 2010a).

2.8. Microarray Data Analysis

Microarray was performed using Affymetrix Human Gene 2.0 ST Array. Genes with a false discovery rate (FDR) of $<5\%$ and a fold change (FC) >1.5 were considered differentially expressed. Heat maps illustrating expression patterns of genes were generated using pheatmap package in R. Results have been deposited at GEO (GSE95482).

2.9. ELISA

Albumin (BETHYL Laboratories, Inc.) in the cell culture medium, ANGPTL3 (R&D DANL30) concentrations in the cell culture medium and plasma were determined using commercial kits.

2.10. Statistical Analysis

Values are shown as mean ± standard error of the mean. Data were analyzed using two-tailed unpaired Student's *t*-test (Graphpad Prism 7). A p value < 0.05 was considered statistically significant.

3. Results

3.1. Generation of Subject-Specific iPSCs and Differentiation into HLCs

Two TD patients, one homozygous for the E1005X nonsense mutation (TD-1) and one compound heterozygote at S2046R/K531N (TD-

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