AMGEN SWISS LIPID RESEARCH AWARD application

Paolo Zanoni, MD

Institute of Clinical Chemistry, University Hospital Zürich, Switzerland

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Validation of new potential receptors and molecular regulators of holo-HDL uptake in the liver

Overview of the research area

Atherosclerosis is characterized by the progressive accumulation of cholesterol in the intima of the arteries (Libby, Ridker, & Hansson, 2011) and leads to cardiovascular disease. Plasma levels of high density lipoproteins (HDL)-associated cholesterol are inversely associated with cardiovascular disease in the general population (Gordon, Castelli, Hjortland, Kannel, & Dawber, 1977)(Assmann, Schulte, Von Eckardstein, & Huang, 1996). Many mechanisms have been proposed to explain the atheroprotective effect of HDL (Kontush et al., 2014). The best described one is the ability of HDL to extract cholesterol from peripheral tissues, including macrophages in the intima of the arteries, and to deliver it to the liver for biliary excretion (Lewis & Rader, 2005; Rader, Alexander, Weibel, Billheimer, & Rothblat, 2009)(von Eckardstein, Nofer, & Assmann, 2001). Part of the delivery of cholesterol to the hepatocyte is operated by the Scavenger Receptor Class B type I (SR-BI). SR-BI has the ability to mediate the selective uptake of HDL cholesterol, while the rest of the HDL particle is released into the extracellular compartment (Acton et al., 1996)(Brundert et al., 2005). Complete loss-of-function SR-BI mutations are associated with increased cardiovascular risk in humans (Zanoni et al., 2016). Besides selective cholesterol uptake though, some HDL particles are endocytosed by hepatocytes as whole particles (Röhrl & Stangl, 2013). This holoparticle uptake has also been described in extrahepatic tissues, but its molecular basis is little understood. The only unequivocally characterized HDL-holoparticle receptor is the cubilin/megalin complex which is only expressed in the kidney and mediates the tubular reabsorption of many proteins including apoA-I(Hammad, 2000)(Hammad et al., 1999)(Aseem et al., 2014). In hepatocytes and endothelial cells, apoA-I activates the ectopic beta-ATPase/purinergic receptor axis which signals to an as yet unknown downstream endocytic HDL receptor(Martinez et al., 2003)(Jacquet et al., 2005). CD36 and SR-BI have also been discussed as endocytic HDL receptors but the data are controversial (Brundert et al., 2011)(Silver, Wang, Xiao, & Tall, 2001). To discover new genes involved in holoparticle uptake of HDL we performed a microscopy-based genome-wide siRNA screen.

Achieved results

We tested the effect of approximately 68000 different siRNAs (3 siRNAs per gene) on the uptake of HDL fluorescently labelled in their protein moiety (fl-HDL) by the human hepatocarcinoma cell line Huh-7. Briefly, 72 hours after siRNA transfection, the cells were exposed to fl-HDL for 4 hours, followed by fixation. The cells were then imaged with an automated microscope. The images were

segmented to identify the nuclei (stained with Hoechst33258) and the HDL-containing vesicles and their features (e.g. vesicle intensity and cytoplasm granularity) were measured automatically. This image analysis phase was followed by a data analysis phase aimed to identify the genes that, when knocked down, displayed the largest effect on HDL uptake. During the QC phase we determined that both the transfection efficiency throughout the screening and the assay windows between negative and positive controls were excellent (yielding Z'-factors constantly above 0.5). After the QC step, the data for each feature were normalized by batch, microscope, plate and well, followed by robust Z-score normalization. The redundant siRNA activity test (RSA) was then applied to rank the 3 siRNA molecules against each gene based on their combined experimental effect. Many siRNAs were found to affect the uptake of HDL yielding hit lists containing 50 to 200 genes according to the assay feature that was analyzed and to the p-value cutoff chosen. Interestingly, when functional interaction networks were generated with these hits, many of the top hits display a high degree of functional interrelation. Furthermore, a Gene Ontology analysis revealed a significant enrichment for endocytosis and intracellular trafficking, supporting the idea that the top hits that were identified reflect the actual biology of HDL uptake and do not represent a list of randomly selected genes.

Research question

Due to the inherent limits of the siRNA technology, the hits require further validation. We will start by filtering the top hits based on their ability to fulfill as many as possible of the following criteria:

a) Strong response to all 3 different siRNAs targeting the candidate gene and subsequent low RSA p-value.

b) Consistent effect by different image analysis features.

c) Have a GWAS signal for plasma HDL cholesterol levels or CHD risk in publicly available GWAS data(Teslovich et al., 2010).

d) Are coregulated/coexpressed with genes known to be involved in lipid metabolism based on large scale microarray and RNAseq data available through databanks such as Genevestigator (https://genevestigator.com/gv/).

e) Have sequences in their promoter region that are recognized by transcription factors involved in the regulation of cellular lipid metabolism (e.g. sterol responsive elements).

Within the next 2 months we will analyze the screening data for consistency and robustness and apply various bioinformatic approaches to integrate the data with other publicly available data to define 20 top candidates. I hereby search support by the AMGEN SWISS LIPID RESEARCH AWARD to pursue the following specific questions:

1. Which of the top 20 candidate genes result in altered HDL holoparticle uptake when targeted with siRNA molecules other than the ones used in the screening?

2. Is the effect of robustly modulating genes specific for HDL?

3. How do verified top candidates modulate HDL binding, internalization and resecretion in hepatocytes?
4. Does overexpression of specifically and robustly modulating genes induce a change in binding and uptake of HDL *in vitro*?

Work plan

We plan to answer the research questions above with the following stepwise experiments:

1. A custom-made array of siRNA against the top screening hits obtained from a different vendor will be generated. The HDL uptake assay used in the main screen will be repeated using the new siRNA molecules. These confirmation experiments will be performed with a high number of replicates to ensure reproducibility. Furthermore, different concentrations of each siRNA will be employed in order to generate dose-response curves. Knockdown efficiency will be measured by real time PCR for each candidate gene.
2. We will make sure that HDL rather than the label has been the target. To this end I will be studying the effect of the most efficient siRNAs on the uptake of ^{125}I-labelled HDL, the gold standard for studying lipoprotein endocytosis endocytosis (Cavelier, Ohnsorg, Rohrer, & von Eckardstein, 2011; L Rohrer et al., 2009; Lucia Rohrer et al., 2006). I will also compare the effects of siRNAs on the uptake of native and reconstituted HDL. It may become important to also test other plasma proteins and lipoproteins known to be taken up by the liver, e.g. LDL, transferrin, transcobalamin, asialoglycoproteins...
3. Differences in the intracellular accumulation of fluorescent HDL can result from differences in binding, uptake, degradation, or resection of HDL. We will hence use the radiolabeled HDL to investigate whether candidate genes affect binding, uptake, degradation or re-secretion of HDL (Cavelier, Ohnsorg, Rohrer, & von Eckardstein, 2011; L Rohrer et al., 2009; Lucia Rohrer et al., 2006).
4. For the genes that based on the publicly available structural information and data from step 3 are potential cell surface receptors, transcription factors or any other proteins whose effect could be dependent on the amount of protein itself, we will also perform overexpression experiments followed by studying the binding, cell association, degradation and resecretion of ^{125}I-HDL.