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Mutations in the SORT1 gene are unlikely to cause autosomal dominant hypercholesterolemia

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ABSTRACT

Objective: To study whether mutations in the SORT1 gene could be a cause of autosomal dominant hypercholesterolemia and to study the effect of sortilin on the binding and internalization of low density lipoprotein (LDL).
Methods: 842 unrelated hypercholesterolemic subjects without mutations in genes known to cause autosomal dominant hypercholesterolemia, were screened for mutations in the SORT1 gene by DNA sequencing. Transfections of wild-type or mutant SORT1 plasmids in HeLa T-REx cells and the use of siRNA were used to study the effect of sortilin on the number of cell-surface LDL receptors and on the binding and internalization of LDL.
Results: A total of 45 mutations in the SORT1 gene were identified of which 15 were missense mutations. Eight of these were selected for in vitro studies, of which none had a major impact on the amount of LDL bound to the cell surface. There was a positive correlation between the amount of sortilin on the cell surface and the amount of LDL bound. The observation that a mutant sortilin which is predominantly found on the cell surface rather than in post-Golgi compartments, bound very high amounts of LDL, indicates that sortilin does not increase the binding of LDL through an intracellular mechanism. Rather, our data indicate that sortilin binds LDL on the cell surface.
Conclusion: Even though sortilin binds and internalizes LDL by receptor-mediated endocytosis, mutations in the SORT1 gene are unlikely to cause autosomal dominant hypercholesterolemia and may only have a marginal effect on plasma LDL cholesterol levels.

1. Introduction

Sortilin is a Type 1 transmembrane protein which acts as a multiligand receptor [1,2]. It is predominantly found in the trans-Golgi network and in early endosomes and less than 10% is on the plasma membrane [3]. The main function of sortilin is to transport ligands between the trans-Golgi network and the early endosomes, but also to bind and internalize various ligands across the cell membrane by receptor-mediated endocytosis [2,4].

Sortilin is a proprotein encoded by the SORT1 gene on chromosome 1p13.3 [1]. The 800 amino acid precursor is cleaved by furin in the Golgi apparatus to release a 44 amino acid propeptide [1]. The mature protein consists of a vacuolar protein sorting 10 protein (Vps10p) domain which is the ligand-binding domain, a 22 amino acid transmembrane domain and a 53 amino acid cytoplasmic domain [1]. The Vps10p domain consists of a 10-bladed β-propeller that is also found in four other structurally related proteins involved in intracellular transport. It is the tunnel of the β-propeller that interacts with ligands, but this interaction is prevented by the uncleaved prodomain which masks the tunnel until sortilin reaches the Golgi apparatus [2]. Thus, it is only after the prodomain has been released in the Golgi apparatus, that sortilin may bind ligands for transport to the early endosomes. The cytoplasmic domain contains several motifs that interact with adapter proteins to shuttle sortilin from the trans-Golgi network to the endosomes and to concentrate sortilin in clathrin-coated pits on the cell membrane [1].

Whereas, sortilin first was implicated in intracellular transport in neurons of the central and peripheral nervous system, it also functions in several other tissues, such as the liver, and has recently been found to play a role in lipid metabolism. The first clues for a role in lipid metabolism came from genome-wide association studies showing that a locus on chromosome 1p13.3 was associated with levels of low density lipoprotein (LDL) cholesterol and the risk of myocardial infarction [5,6].

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This locus on chromosome 1p13.3 contains the four genes cadherin EGF LAG seven-pass G-type receptor 2 (CELSR2), proline/serine-rich coiled-coil 1 (PSRC1), myosin binding protein H-like (MYBPHL) and SORT1, and subsequent studies have shown that it is SORT1 that affects lipid metabolism [7]. A key finding in this respect was that the single nucleotide polymorphism rs12740374 between the CELSR2 and PSRC1 genes alters a predicted binding site for the transcription factor CCAAT/enhancer binding protein (C/EBP), with the major allele disrupting the binding site and the minor allele creating it [7]. As a consequence, the minor allele increases the expression of the SORT1 gene [7].

The effect of sortilin on very low density lipoprotein (VLDL) synthesis, as determined by overexpression and knockdown studies in mouse models, is conflicting [4,7–9]. Musunuru et al. [7] found that sortilin reduced the synthesis of VLDL and thereby reduced the levels of LDL cholesterol, whereas Kjolby et al. [8] found that sortilin increased the synthesis of VLDL and thereby increased the levels of LDL cholesterol. Differences in the genetic make-up of the two mouse models have been suggested as a possible explanation for this discrepancy. Moreover, Linsel-Nitschke et al. [10] found increased amounts of LDL internalized in HEK293 cells transfected with a SORT1 plasmid. However, whether this reflects sortilin-mediated binding and internalization of LDL at the cell surface or if sortilin increases the number or functionality of the LDL receptors by some intracellular effect, is unknown. Thus, the exact mechanism for the effect of sortilin on lipid metabolism remains to be determined.

Mutations in the LDL receptor (LDLR) gene cause familial hypercholesterolemia which is an autosomal dominant form of hypercholesterolemia characterized by xanthomas and premature coronary heart disease [11]. From the data of Linsel-Nitschke et al. [10] showing that overexpression of sortilin increased the amount of LDL internalized, one may speculate that mutations in the SORT1 gene could reduce the amount of LDL internalized and thereby cause autosomal dominant hypercholesterolemia.

In this study we have screened severe hypercholesterolemic subjects without mutations in genes known to cause autosomal dominant hypercholesterolemia, for mutations in the SORT1 gene. Moreover, the impact of wild-type (WT) SORT1 and selected mutations in the SORT1 gene on the binding and internalization of LDL, has been studied.

2. Material and methods

2.1. Subjects

842 unrelated hypercholesterolemic subjects referred for genetic testing with respect to familial hypercholesterolemia were included. None of these subjects were heterozygous for a mutation in the LDLR gene or in the proprotein convertase subtilisin/kexin type 9 (PCSK9) gene or were heterozygous for mutation R3500Q in the apolipoprotein B-100 gene, as determined by standard Sanger DNA sequencing. There were 537 females and 305 males and their mean (±SD) age was 54.5 (±11.6) years. Their mean (±SD) levels of total serum cholesterol before lipid-lowering therapy was started, were 10.2 (±1.3) mmol/l.

2.2. DNA sequencing of the SORT1 gene

A total of 19 amplicons that together spanned the 20 exons with flanking intron sequences of the SORT1 gene were amplified by polymerase chain reaction and subjected to DNA sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA) and analyzed on a 3730XL Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). The primer sequences are listed in Supplementary Table S1. The conditions for thermal cycling are available upon request.

2.3. Cell culture

HeLa T-REx cells (Invitrogen, Carlsbad, CA) were cultured in Modified Eagle's medium (PAA Laboratories GmbH, Pasching, Austria) containing streptomycin (50 μg/ml), penicillin (50 U/ml), L-glutamine (2 mM) and 10% fetal calf serum (Invitrogen), in a humidified atmosphere (37 °C, 5% CO_2).

2.4. Mutageneses and transfections

The human SORT1 cDNA (OriGene, Rockville, MD) was cloned into pIRES2-AcGFP1 plasmid (Clontech Laboratories, Inc., Mountain View, CA) using SacI and XmaI restriction sites to generate pIRES2-WT-SORT1. The pIRES2-AcGFP1 is a bicistronic vector where the gene of interest and the Aequoirea coerulescens green fluorescent protein 1 (AcGFP1) gene are translated from a single mRNA. This allows selection of only transfected cells for the flow cytometric analyses.

pIRES2-WT-SORT1 was used as a template for mutagenesis to generate mutant SORT1 plasmids. The primer sequences used for mutageneses are listed in Supplementary Table S2 and mutagenesis was carried out using QuickChange XL Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The integrity of the plasmids was confirmed by DNA sequencing.

HeLa T-REx cells were transiently transfected using FuGENE HD (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

Small interfering (si)RNAs targeting the SORT1 gene or the LDLR gene as well as control siRNAs were obtained from Qiagen (Qiagen GmbH, Hilden, Germany) and transfections were performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

2.5. Western blot analysis of sortilin

Western blot analysis was carried out essentially as previously described [11]. Briefly, cell lysates were obtained and run on 4–20% Tris–HCl Criterion Precast Gels (Bio-Rad, Hercules, CA) and blotted onto Immuno-Blot PVDF Membranes (Bio-Rad, Hercules, CA). The membranes were immunostained with a rabbit anti-sortilin antibody (Abcam, Cambridge, UK).

2.6. Quantitation of the amount of cell-surface sortilin and LDLR

The amount of cell-surface LDLR on HeLa T-REx cells was determined by flow cytometry as previously described [12]. Briefly, the cells were cultured in medium containing 5 mg/ml lipoprotein-deficient serum for 24 h to increase the expression of the LDLR. The cells were then incubated with a mouse anti-LDLR monoclonal antibody IgG-C7 (Progen Biotechnik GmbH, Heidelberg, Germany) or a goat anti-sortilin antibody (R&D Systems, Inc., Minneapolis, MN) for 1 h at 4 °C, washed and incubated with Alexa Fluor® 647 goat anti-mouse IgG or Alexa Fluor® 647 donkey anti-goat IgG (Molecular Probes, Eugene OR), respectively for 30 min at 4 °C.

2.7. Quantitation of the amount of LDL bound and internalized

The amount of LDL bound and internalized in HeLa T-REx cells was determined by flow cytometry. Briefly, the cells were cultured in medium containing 5 mg/ml lipoprotein-deficient serum for 24 h to increase the expression of the LDLR. The cells were then washed and incubated with 10 μg/ml of 1,1′