Atherosclerosis 238 (2015) 304–312

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Activity-associated effect of LDL receptor missense variants located in the cysteine-rich repeats

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ARTICLE INFO

Article history:
Received 23 July 2014
Received in revised form
9 December 2014
Accepted 13 December 2014
Available online 20 December 2014

Keywords:
LDLR
Mutations
Familial hypercholesterolemia
Ligand binding domain
Mutation class defect

ABSTRACT

Background: The LDL receptor (LDLR) is a Class I transmembrane protein critical for the clearance of cholesterol-containing lipoprotein particles. The N-terminal domain of the LDLR harbours the ligand-binding domain consisting of seven cysteine-rich repeats of approximately 40 amino acids each. Mutations in the *LDLR* binding domain may result in loss of receptor activity leading to familial hypercholesterolemia (FH). In this study the activity of six mutations located in the cysteine-rich repeats of the *LDLR* has been investigated. **Methods**: CHO-*ldlA7* transfected cells with six different *LDLR* mutations have been used to analyse *in vitro* LDLR expression, lipoprotein binding and uptake. Immunoblotting of cell extracts, flow cytometry and confocal microscopy have been performed to determine the effects of these mutations. *In silico* analysis was also performed to predict the mutation effect. **Results and conclusion**: From the six mutations, p.Arg257Trp turned out to be a non-pathogenic LDLR variant whereas p.Cys116Arg, p.Asp168Asn, p.Asp172Asn, p.Arg300Gly and p.Asp301Gly were classified as binding-defective LDLR variants whose effect is not as severe as null allele mutations.

1. Introduction

Familial Hypercholesterolemia (FH; MIM#143890) is an autosomal dominant disorder causing premature coronary heart disease (CHD) [1] that is characterized by increased plasma LDL cholesterol, tendon xanthomas, deposits of cholesterol in peripheral tissues and accelerated atherosclerosis. FH has a homozygous frequency of 1:1,000,000, and its heterozygous frequency has recently been estimated to be as high as 1/200 in the general population [2,3], suggesting that the disease is heavily underdiagnosed and undertreated. FH is mainly due to mutations in the LDL receptor (LDLR; MIM# 606945) gene, which is responsible for the uptake of LDL particles into cells [1].

The LDLR is a modular protein that combines five different domains: the ligand binding domain, the EGF-like module that contain a 280 amino acid β-propeller, the O-glycosylated domain and the transmembrane and cytoplasmic domains. The binding domain of LDLR comprises ~40-amino acid long cysteine-rich repeats in tandem, structured in seven discrete extracellular modules (R1–R7), which are responsible for the binding and release of its lipoprotein ligands [4]. After binding, the LDLR-lipoprotein complex is internalized through clathrin-coated pits and traffics to endosomes, where lipoprotein cargo is released [5,6]. Lipoproteins are subsequently degraded in lysosomes, while the LDLR recycles back to the cell surface for further rounds of lipoprotein uptake.

Nowadays more than 1300 different variants have been described in the LDLR gene [7], not all of them pathogenic. According to the nature and location of the mutations within the *LDLR* and to the phenotypic effects on the protein, mutations have been divided into five different classes [8]: Class 1: no detectable LDLR synthesis; Class 2: defective LDLR transport; Class 3: impaired LDL to LDLR binding; Class 4: no LDLR/LDL internalization due to defective clustering in clathrin-coated pits; and Class 5: no LDLR recycling.

The fact that several missense variants of the *LDLR* found in FH patients have been shown not to be the actual cause of the disease [9,10] indicates that every detected variant needs to be functionally characterized in order to determine its severity, if any.

The aim of this study was to analyse the impact on the LDLR activity of six missense variants located in the ligand binding domain of the protein, and previously found in FH patients. The sequence variations studied predict the following amino acid

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http://dx.doi.org/10.1016/j.atherosclerosis.2014.12.026
0021-9150/

changes in the LDLR: p.Cys116Arg, p.Asp168Asn, p.Asp172Asn, p.Arg257Trp, p.Arg300Gly and p.Asp301Gly. The effects on LDLR expression, binding capacity and uptake were studied by Western blot, flow cytometry and confocal microscopy in a transfected *LDLR*-defective Chinese hamster ovary (CHO) cell line.

2. Materials and methods

2.1. Selection of variants

The selection of the six missense *LDLR* variants was based on two criteria: to have been previously found in FH patients and to be possibly associated to a binding defect. To cover the first criteria we selected variants previously described by other authors in FH patients that have also been found in at least one FH index case by LIPOchip® platform [11] or by SEQPRO LIPO RS® platform in Progenika Biopharma (Derio, Spain), both platforms with the CE mark. Just two of the variants have been found in large population studies, p.Asp168Asn and p.Arg257Trp, that were coded as rs200727689 and rs200990725, respectively in the NCBI SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?geneId=3949). Both of them have a very low frequency, p.Asp168Asn has been found in the EVS database (http://evs.gs.washington.edu/EVS/) with a MAF(%) = 0.0154 and p.Arg257Trp in the 1000 genomes database (http://browser.1000genomes.org.) with a MAF(%) = 0.001. The characteristics of the selected variants are compiled in Table 1 and their location within the protein is shown in Fig. 1.

2.2. Site-directed mutagenesis

Plasmids carrying the *LDLR* variants were constructed by Innoprot (Derio, Spain) as described in Online Supp. Data.

2.3. Cell culture and transfection

LDLR-deficient CHO cell line *ldl*A7 (CHO-*ldl*A7) (kindly provided by Dr. Monty Krieger, Massachusetts Institute of Technology, Cambridge, MA) was cultured in Ham's F-12 medium supplemented with 5% FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. CHO-*ldl*A7 cells were plated into 6- or 24-well culture plates, and transfected with plasmids carrying the *LDLR* variants using Lipofectamine® LTX and Plus™ Reagent (Invitrogen) according to the manufacturer's instructions. Transfected cells were maintained in culture during 48 h to achieve maximal LDLR expression.

Table 1
Characteristics of *LDLR* variants included in the study.

	Location	cDNA (HGVS)	Protein (HGVS)	LDLR domain	Reference
Positive controls	Exon 3 and 4	c.191-¿_694+?del	p.[L64S];[S65_A232del]	Ligand binding; ΔR2-R4	[9,22].
	Exon 3	c.261G > A	p.W87*	Ligand binding; ΔR2	[23].
Single nucleotide variants	Exon 4	c.346T > C	p.Cys116Arg	Ligand binding; R3	[24].
	Exon 4	c.502G > A	p.Asp168Asn	Ligand binding; R4	[25].
	Exon 4	c.514G > A	p.Asp172Asn	Ligand binding; R4	[26].
	Exon 5	c.769C > T	p.Arg257Trp	Ligand binding; R6	[27,28].
	Exon 6	c.898A > G	p.Arg300Gly	Ligand binding; R7	[29,30].
	Exon 6	c.902A > G	p.Asp301Gly	Ligand binding; R7	[28,31,32].

2.4. Western blot analysis

Cell lysates were prepared, protein concentration determined, and fractionated by electrophoresis as described in Online Supp. Data.

2.5. Lipoprotein isolation

LDL and VLDL were isolated from blood samples of healthy individuals in a two step centrifugation as described in Online Supp. Data.

2.6. Lipoprotein labelling

LDL and VLDL were labelled with FITC as previously described [12]. Briefly, lipoproteins (1 mg/mL) in 0.1 M $NaHCO_3$ (pH 9.0) was mixed with 10 μl/mL FITC (2 mg/mL in dimethyl sulfoxide). The mixture was gently mixed by slow rocking at room temperature for 2 h. The unreacted dye was removed by gel filtration on a Sephadex G-25 column equilibrated with PBS EDTA-free buffer. All fractions were assayed for protein content with bovine serum albumin as standard (Pierce BCA protein assay, Pierce).

2.7. Quantification of LDLR activity by flow cytometry

Transfected CHO-*ldl*A7 cells were grown in 24-well culture plates. 48 h after transfection, cells were incubated for 4 h, at 37 °C or at 4 °C with 20 μg/mL FITC-LDL to determine LDLR activity or LDL-LDLR binding, respectively. After incubation with FITC-LDL, CHO-*ldl*A7 cells were washed twice in PBS-1%BSA, fixed on 4% formaldehyde for 10 min and washed again twice with PBS-1%BSA. To determine the amount of internalized LDL, Trypan blue solution (Sigma–Aldrich, Steinheim, Germany) was added directly to the samples to a final concentration of 0.2%, eliminating the extracellular signal due to the non-internalized LDL-LDLR complexes. Measurement of VLDL was performed by incubation of cells with 20 μg/mL FITC-VLDL for 4 h, at 37 °C as described for LDL. Fluorescence intensities were measured by FACS, in a Facscalibur Flow cytometer according to the manufacturer instructions as previously described [9]. For each sample, fluorescence of 10,000 events was acquired for data analysis. All measurements were performed at least in triplicate.

2.8. Quantification of LDLR expression by flow cytometry

To determine LDLR cell surface expression by FACS, transfected CHO-*ldl*A7 cells grown during 48 h were incubated with a mouse primary antibody anti-LDLR (1:100; 2.5 mg/L; Progen Biotechnik GmbH) for 1 h, at room temperature, then washed twice with PBS-1%BSA and incubated with secondary antibody Alexa Fluor 488-conjugated goat anti-mouse IgG (1:100; Molecular Probes). For each sample, fluorescence of 10,000 events was acquired for data analysis. All measurements were performed at least in triplicate.

2.9. Confocal laser scanning microscopy (CLSM)

CLSM was used to analyse LDL-LDLR binding and LDL uptake in *LDLR* transfected CHO-*ldl*A7 cells. Briefly, cells were plated in coverslips and then transfected with the *LDLR* containing plasmids and cultured for 48 h, at 37 °C in 5% CO_2. Then the medium was removed and coverslips washed twice with PBS-1%BSA. To determine LDL-LDLR binding and LDL uptake, non-labelled lipoproteins