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Identification of mutations in the apolipoprotein B-100 gene and in the PCSK9 gene as the cause of hypocholesterolemia

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ABSTRACT

Background: Characterization of the normally occurring mutations as the cause of hypocholesterolemia may increase our understanding of the normal lipid metabolism.
Methods: DNA from 93 unrelated hypocholesterolemic subjects with a mean (±SD) value for total serum cholesterol of 3.3 (±0.5) mmol/l) were subjected to DNA sequencing of the individual exons of the apolipoprotein B-100 (apoB-100) gene and of the proprotein convertase subtilisin/kexin 9 (PCSK9) gene. The same analyses were also performed in 23 unrelated subjects with autosomal dominant hypercholesterolemia who had unusually low levels of total serum cholesterol.
Results: Of the 93 hypocholesterolemic subjects, 9 subjects (9.7%) were heterozygous for a truncating mutation in the apoB-100 gene and six subjects (6.5%) were heterozygous for a loss-of-function mutation in the PCSK9 gene. Of the 23 subjects with autosomal dominant hypercholesterolemia, four subjects (17.4%) were heterozygous for mutations in the apoB-100 gene.
Conclusion: Truncating mutations in the apoB-100 gene are slightly more common as the cause of hypocholesterolemia compared to loss-of-function mutations in the PCSK9 gene. It appears that mutations in the apoB-100 gene may completely normalize the lipid profile in subjects with autosomal dominant hypercholesterolemia, whereas loss-of-function mutations in the PCSK9 gene do not have a sufficient cholesterol-lowering capacity.

1. Introduction

Approximately 70% of plasma cholesterol is carried in low density lipoprotein (LDL) [1] and an increased level of LDL cholesterol is a major risk factor for coronary heart disease [2]. LDL is synthesized as a metabolic product of very low density lipoprotein (VLDL), and the level of LDL cholesterol is affected both by environmental and genetic factors [2]. The genetic factors constitute genes encoding proteins involved in the metabolism of LDL. Two key proteins involved in the synthesis and secretion of VLDL are apolipoprotein B-100 (apoB-100) and microsomal triglyceride transfer protein (MTP) [3]. Mutations in the apoB-100 gene or in the MTP gene are established causes of autosomal dominant or autosomal recessive hypocholesterolemia, respectively. The key protein involved in the clearance of LDL is the cell-surface LDL receptor [4]. LDL binds to the LDL receptor and is subsequently internalized by receptor-mediated endocytosis [4]. ApoB-100, which is the sole apolipoprotein of LDL, is the constituent of LDL interacting with the LDL receptor. Thus, the normal clearance of LDL by the LDL receptor is dependent upon normal function of both the LDL receptor and of apoB-100. Accordingly, mutations in the LDL receptor gene and in the apoB-100 gene have been found to cause autosomal dominant hypercholesterolemia. Mutations in the LDL receptor gene cause familial hypercholesterolemia [4], whereas mutations in the apoB-100 gene cause familial defective apoB-100 [5].

The number of LDL receptors is post-transcriptionally regulated by proprotein convertase subtilisin/kexin 9 (PCSK9) [6,7]. Secreted PCSK9 performs this function by binding to the epidermal growth factor (EGF) repeat A of the LDL receptor [10]. Somehow, bound PCSK9 disrupts the normal recycling of the LDL receptor leading to its intracellular degradation [8–10], Mutations in the PCSK9 gene may cause hypo- or hypercholesterolemia due to decreased or increased LDL receptor-degrading activity, respectively [11]. Mutations in the PCSK9 gene causing hypocholesterolemia are referred to as loss-of-function mutations, and cause low levels of LDL cholesterol due to increased number of LDL receptors. Conversely, mutations in the PCSK9 gene causing hypercholesterolemia are referred to as gain-of-function mutations, and cause high levels of LDL cholesterol due to decreased number of LDL receptors.

Characterization of the molecular mechanisms underlying hyper- or hypocholesterolemia have provided important information about the normal lipid metabolism. The major focus in this respect has been on identifying mutations causing elevated levels of LDL cholesterol. Subjects possessing such mutations have an increased risk of coronary heart disease, and identifying an underlying mutation may form the

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basis for preventive lipid-lowering measures as well as for screening of family members.

In this study we have identified mutations causing hypocholesterolemia in healthy subjects by performing DNA sequencing of the 29 exons with flanking intron sequences of the apoB-100 gene, of the 12 exons with flanking intron sequences of the PCSK9 gene and exon 7 of the LDL receptor gene.

2. Materials and methods

2.1. Subjects

The 93 unrelated hypocholesterolemic subjects (45 females, 48 males) were identified through a screening program of 40-year old subjects in Oslo or through cascade genetic screening for familial hypercholesterolemia. They were ostensible healthy with a mean (±SD) age of 36.7 (±11.1) years. They had a value for total serum cholesterol between 1.5 mmol/l and 4.2 mmol/l (corresponding to the 14. percentile in healthy adult Norwegians), with a mean (±SD) value of 3.3 (±0.5) mmol/l (corresponding to the first percentile in healthy adult Norwegians). The values for LDL cholesterol were between 0.3 mmol/l and 2.9 mmol/l, with a mean value of 1.8 (±0.5) mmol/l. In addition, 23 unrelated subjects with autosomal dominant hypercholesterolemia due to mutations in the LDL receptor gene ($n=22$) or in the PCSK9 gene ($n=1$), who presented with unusually low levels of total serum cholesterol, were included. This group consisted of 10 females and 13 males, and the mean (±SD) age was 24.3 (±10.7) years. Without any lipid-lowering therapy they had a value for total serum cholesterol between 2.6 mmol/l and 6.1 mmol/l, with a mean (±SD) value for total serum cholesterol of 5.1 (±0.9) mmol/l. The values for LDL cholesterol were between 1.0 mmol/l and 4.1 mmol/l, with a mean (±SD) value of 3.3 (±0.9) mmol/l. Measurements of serum lipids were performed as part of routine diagnostic measurements at the Departments of Biochemistry at Norwegian hospitals. Values for LDL cholesterol were calculated by the use of the formula of Friedewald et al. [12] which requires that the blood samples are obtained in the fasting state. However, because some uncertainties exist regarding the fasting state of a proportion of the subjects, some caution should be exerted when interpreting the values for LDL cholesterol. This applies also to the values for triglycerides. Written informed consent was obtained from all subjects.

DNA sequencing was performed on DNA extracted from EDTA-containing blood by the use of a BioRobot EZ1 (Qiagen GmbH, Hilden, Germany). DNA sequencing of the promoter region and of the 29 exons with flanking intron sequences of apoB-100 gene, of the 12 exons with flanking intron sequences of the PCSK9 gene and of exon 7 with flanking intron sequences of the LDL receptor gene, was performed by the use of version 3.1 of Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) on exon-containing PCR products. A total of 46 PCR products from the coding sequence of the apoB-100 gene were sequenced. The sequencing reactions were analyzed on a Genetic Analyzer 3730 (Applied Biosystems, Foster City, CA) by the use of SecScape version 2.5 software (Applied Biosystems, Foster City, CA). The numbering of nucleotides and codons in the apoB-100 gene was according to convention. Nucleotide 1 of cDNA was nucleotide A of the ATG initiation codon, whereas codon 1 was the first codon of the mature apoB-100 after the 27 residue signal peptide has been cleaved off. For the PCSK9 gene the numbering followed the recommendations [13] that nucleotide 1 is A of the ATG initiation codon and codon 1 is the ATG initiation codon. The primer sequences and conditions for the thermal cyclings are available upon request.

3. Results and discussion

Of the 93 unrelated subjects with hypocholesterolemia, eight (8.6%) were heterozygous for a non-sense mutation or a frame-shift mutation in the apoB-100 gene (Table 1). Thus, in these subjects the mutation in the apoB-100 gene is expected to result in truncated apoB. One subject was heterozygous for a splice-site mutation. Five subjects (5.4%) were heterozygous for a 3 bp in-frame deletion in the apoB-100 gene, which deletes codon 2186 encoding aspartic acid. Finally, six subjects (6.5%) were heterozygous for loss-of-function mutations in the PCSK9 gene.

Table 1
Mutations in the apoB-100 gene as the cause of truncated apoB or as the cause of an in-frame deletion of one amino acid among 93 unrelated subjects with hypocholesterolemia

Mutation	Nucleotide change	Exon/ intron	Truncated apoB	No. of patients	Total serum cholesterol (mmol/l)
537+1, G>T	537+1, G>T	5	ApoB-3	1	4.2
1250delT	1250delT	10	ApoB-9	1	3.3
4186–4187delGT	4186–4187delGT	25	ApoB-30	1	1.5
4800–4801insC	4800–4801insC	26	ApoB-34	1	2.6
Y1719X	5238, T>A	26	ApoB-38	1	2.8
6636–6638delTGA	6636–6638delTGA	26	ΔD2186	5	2.8, 2.9, 3.2, 3.3, 3.6
7158delA	7158delA	26	ApoB-52	1	3.8
R2486X	7537, C>T	26	ApoB-55	1	3.1
8771delC	8771delC	26	ApoB-64	1	3.1
10728dupC	10728dupC	26	ApoB-79	1	2.5

The number of patients with the individual mutations and the values for total serum cholesterol in these patients are indicated.

The splice site mutation 537 1, G>T in intron 5 of the apoB-100 gene has previously been found to cause hypocholesterolemia in a Caucasian subject residing in the United States [14]. Because, the invariant G of the +1 consensus splice site is affected, the mutation is expected to affect RNA splicing. If the mutation causes exon 5 to be skipped from mRNA, the two last nucleotides of exon 6 and the first nucleotide of exon 7 will constitute a UGA stop codon. This will result in a truncated protein of only 152 amino acids. If the mutation causes intron 5 to be retained in mRNA, a UAG stop codon will be reached four triplets into intron 5. This will result in a truncated protein of only 155 amino acids. Both these proteins will only be 3% of the size of the normal, mature apoB-100 of 4536 amino acids, and will be designated apoB-3 according to the centile nomenclature. However, none of these truncated proteins will appear in plasma since truncated apoBs which are shorter than 27% of normal have not been found in plasma [15]. The mechanisms behind this observation could be the failure of the short apoBs to be produced in sufficient amounts or the failure to facilitate the synthesis of a lipoprotein particle [16]. Rapid catabolism could also contribute to the failure to observe short truncated apoBs in plasma [16].

The six frame-shift mutations, one of the two nonsense mutations and the in-frame deletion in the apoB-100 gene are novel mutations. 1250delT in exon 10 results in a frame-shift and a stop codon is reached in codon 418. Thus, a truncated apoB (apoB-9) is produced which is too short to appear on a lipoprotein particle detectable in plasma. The two bp deletion 4186–4187delGT in exon 25 results in a frame-shift and a stop codon is reached in codon 1370. Thus, a truncated apoB (apoB-30) is produced which may just be large enough to be incorporated into a lipoprotein particle present in plasma. If so, the apoB-30-containing lipoprotein particle is expected have a density in the high density lipoprotein (HDL) density range [17]. The one bp insertion 4800–4801insC in exon 26 results in a frame-shift and a stop codon is reached in codon 1575. Thus, a truncated apoB (apoB-34) is produced. The apoB-34-containing lipoprotein particle is expected to have a density in the HDL or LDL range [17]. Nonsense mutation Y1719X results in a truncated apoB (apoB-38) which will occur on a lipoprotein particle in the VLDL and LDL density ranges as will larger sized truncated apoBs [17]. The three bp deletion 6636–6638delTGA in exon 26 is an in-frame deletion which deletes codon 2186 which encodes aspartic acid. This was a common mutation found in 5 of the 93 subjects. However, the mutation was also identified in one of 100 unrelated hypercholesterolemic control subjects with values for total serum cholesterol between 7.0 mmol/l and 10.0 mmol/l. None of these hypercholesterolemic subjects had mutations in the LDL receptor gene or in the PCSK9 gene, and none possessed the R3500Q mutation of the apoB-100 gene as the cause of hypercholesterolemia. Even though mutation 6636–6638delTGA was more common among the hypocholesterolemic subjects, it may be a normal genetic variant. Mutation 7158delA in exon 26 causes a frame-shift and a stop codon is reached in codon 2374. Thus, a truncated apoB (apoB-52) is produced. Nonsense mutation R2486X results in a truncated apoB (apoB-55), and has previously been identified in a Caucasian subject residing in the United States [18]. The one bp deletion 8771delC in exon 26 causes a frame-shift and a stop codon is reached in codon 2923. Thus, a truncated apoB (apoB-64) is produced. The one bp duplication 10728dupC in exon 26 causes a frame-shift and a stop codon is reached in codon 3585. Thus, a truncated apoB (apoB-79) is produced.

In our study, nine of the 93 (9.7%) hypocholesterolemic subjects had a mutation leading to truncated apoB. In other studies the corresponding figure ranges from 2% [17,19] to 41% [20]. These differences are assumed to be due to differences in the criteria used for patient selection or in the methods used for mutation detection. Our finding