Inference of a Molecular Defect of Apolipoprotein B in Hypobetalipoproteinemia by Linkage Analysis in a Large Kindred

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Abstract

Heterozygous hypobetalipoproteinemia is characterized by reduced plasma concentrations of LDL cholesterol, total triglycerides, and apo B to < 50% of normal values. The molecular basis of this disorder remains unknown. The phenotype cosegregates with a DNA haplotype of the apo B gene in an Idaho pedigree, with a maximum decimal logarithm of the ratio (LOD) score of 7.56 at a recombination rate of zero. Individuals carrying this haplotype had total cholesterol levels of 96 mg/dl, LDL cholesterol levels of 37 mg/dl, triglycerides levels of 51 mg/dl, and apo B levels of 38 mg/dl. This study strongly suggests that apo B mutations underlie hypobetalipoproteinemia, and demonstrates the power of the candidate gene approach in linkage analysis for unraveling genetic determinants in metabolic disorders of undefined etiology.

Introduction

Two inherited lipoprotein deficiency states, abetalipoproteinemia and hypobetalipoproteinemia (HBLP),¹ share similar clinical and biological features in the homozygous form (1). Affected individuals suffer from fat malabsorption, ataxic neuropathy, atypical retinitis pigmentosa, and acanthocytosis. Chylomicrons, VLDL, and LDL are absent from plasma, and apo B, an obligatory constituent of these three lipid fractions, is not detectable immunologically. Only traces of triglycerides can be identified; cholesterol, essentially confined to the high density lipoprotein (HDL) fraction, typically has a concentration < 50 mg/dl.

The distinction between these two clinical entities rests on the blood lipid profiles of parents, both of whom are obligate heterozygotes and free of clinical manifestations (1). Whereas parents of abetalipoproteinemia patients cannot be distinguished from normal subjects, parents of hypobetalipoproteinemia patients usually have low concentrations of VLDL and LDL, and therefore low plasma concentrations of triglycerides

and cholesterol, the latter best noted in the LDL fraction. No particular abnormality of the lipid particles or of their constituents has been identified for any lipid fraction (1). Investigations of LDL metabolism in such subjects have shown reduced synthesis and normal catabolic rates (2, 3). The molecular basis of this disorder is unknown; any alteration of apo B, lipoprotein assembly, and secretion could account for the clinical and biological manifestations.

Apo B is the major protein constituent of the LDL particle and the ligand for the LDL receptor that clears these particles from the circulation. Recently cDNA and genomic clones of the apo B genes have been isolated in several laboratories (4–8). Screening of random individuals with various restriction enzymes (9, 10), or comparison of DNA sequences in different subjects (11), have identified a number of DNA variants in the genomic region encoding the apo B gene. We have characterized such DNA polymorphisms in a pedigree that includes 17 subjects with heterozygous HBLP. The cosegregation of HBLP with an apo B haplotype strongly suggests that this disorder results from mutations of the apo B gene.

Methods

Selection of subjects. In the course of family studies to identify genetic markers that affect lipoprotein levels and susceptibility to atherosclerosis, an asymptomatic 41-yr-old woman was found to have total cholesterol and triglyceride levels of 80 and 35 mg/dl, respectively, whereas her LDL-cholesterol level was 19 mg/dl. Her spouse and four of her seven offspring were normal, but three offspring had lipid profiles similar to their mother's. The initial analysis of this family, together with the mother's siblings and their living mother, motivated the extension of the pedigree, referred to as pedigree 635. Extension was done by preliminary field analysis of total cholesterol levels on finger-prick specimens, with a portable blood chemistry analyzer (Reflotron; Boehringer Mannheim Diagnostics, Indianapolis, IN), to ascertain other family lineages warranting further investigation.

Blood sampling and lipid and lipoprotein analysis. Blood samples were obtained in EDTA tubes for lipid analysis and in citrated tubes for DNA analysis, from 39 pedigree members who had been fasting for 12–16 h. Lipid and lipoprotein measurements were made on fresh, unfrozen plasma. Total cholesterol, lipoprotein cholesterol, and triglycerides were determined enzymatically using Boehringer Mannheim Biochemicals kits. HDL cholesterol was determined after VLDL and LDL were precipitated by the dextran sulfate method. LDL plus HDL cholesterol were determined on the infranatant after removal of VLDL cholesterol by airfuge ultracentrifugation (Beckman Instruments, Fullerton, CA). LDL cholesterol was determined by subtracting the HDL cholesterol value from the cholesterol in the infranatant. VLDL cholesterol was determined by subtracting the cholesterol in the infranatant from the total cholesterol. apo B concentrations were assayed by RIA with Ventrex kits (Ventrex Laboratories; Portland, ME). apo E phenotypes were determined by isoelectric focusing, as described in reference 12.

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1. *Abbreviations used in this paper:* HBLP, hypobetalipoproteinemia; HDL, high density lipoprotein; LOD, decimal logarithm of the ratio; RFLP, restriction fragment length polymorphism.

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Southern blot analysis. Two cloned segments of the apo B gene, pB8 and pB23, were prepared as described elsewhere (13). The whole plasmids were labeled to a high specific activity (14). From each pedigree member, 5 µg of DNA, prepared as described previously (15), was digested with a 5- to 10-fold excess of restriction endonucleases Xba I, Eco RI, and Msp I (Boehringer Mannheim Biochemicals), electrophoresed in 0.7% agarose gels, and transferred to nylon membranes in a basic transfer buffer (16). Membranes blotted with Eco RI- or Msp I-digested DNA were hybridized with ³²P-labeled pB8, and membranes blotted with Xba I-digested DNA were hybridized with ³²P-labeled pB23. The restriction fragment length polymorphisms (RFLPs) in the apo B gene that can be detected in this manner have been described (9, 13). Prehybridization and hybridization were performed using the solutions and conditions described in reference 17.

Segregation and linkage analysis. The familial distribution of lipid measurements and apo B genotypes were analyzed with the PAP (18) and LINKAGE (19) computer programs. Because the available measurements might not allow the unambiguous identification of HBLP heterozygotes, quantitative measurements were analyzed as such. An autosomal dominant mode of inheritance was assumed for the transmission of HBLP, as well as an arbitrarily low gene frequency for the defective gene. It is assumed that the defective gene affects the mean value of the quantitative phenotypes among carriers and that, conditioned on the genotype at this locus, the quantitative phenotypes follow a multivariate distribution. The distribution of the phenotypes in the pedigree is expressed as a function of the unknown genotypic means and variance-covariances of the traits. The simultaneous segregation of the DNA markers at the apo B locus is expressed as a function of the unknown recombination rate between the HBLP and the apo B loci. The null hypothesis of independent segregation of lipid parameters and the apo B locus was tested by comparing the likelihood of the observations when the recombination fraction was fixed at 0.5 with that obtained when recombination was also estimated, other parameters being estimated in both cases. The decimal logarithm of the ratio thus obtained, or LOD score, is considered significant when it is > 3 (20).

Results

Definition of apo B haplotypes. Xba I, Eco RI, and Msp I RFLP were determined for pedigree members. Linkage disequilibrium between the Msp I and Eco RI sites was such that Eco RI did not contribute additional genotypic information over Msp I genotypes. The Xba I polymorphism, however, allowed definition of the haplotypes necessary to track the inheritance of the apo B gene in this pedigree.

Analysis of the proband's nuclear family. In the family tree shown in Fig. 1, the proband is subject 34, her spouse is subject 33, and her 7 children are subjects 46–52. As reported in Table I, the HBLP phenotype segregated in this family; the proband and three of seven offspring had LDL cholesterol levels ranging between 19 and 36 mg/dl, whereas her spouse and the other four offspring had LDL cholesterol levels ranging from 101 to 119 mg/dl. Southern blot analysis revealed that all four HBLP subjects shared an apo B haplotype not found in their normal relatives. Within this nuclear family, linkage was suggested by a LOD score of 1.8 at a recombination rate of zero.

Analysis of the extended pedigree. The proband's mother, subject 10, did not carry the apo B haplotype that segregated with HBLP in this family. The mutant allele therefore was inherited through the dead father, subject 9. His having had 11 siblings, however, offered the opportunity to detect the occurrence of HBLP in his relatives. The pedigree of Fig. 1 summarizes the branches that were ascertained as described in Methods.

Lipid, apo B, and DNA polymorphism determinations are reported in Table I. For the analysis, cholesterol and apo B measurements were adjusted for age and sex through regression parameters defined in previous studies (21). Because the distribution of triglyceride levels is highly skewed, a logarithmic transformation was applied before the analysis. Simultaneous segregation and linkage analysis, as described in Methods, were performed for each of the four available quantitative measurements. A metabolic disturbance affecting the lipoprotein fraction that contains apo B as a major constituent is likely to alter all four measurements. Three of them, total cholesterol, total triglycerides, and LDL cholesterol, were measured on all sampled subjects. As each parameter involves a measurement error, using them jointly as indicators of the underlying disturbance may increase the power to establish linkage between HBLP and the apo B gene. Results of joint analyses are reported in Table II.

Each of the measurements, except for triglycerides, supported with a LOD score > 3 the hypothesis that the apo B DNA marker segregates with HBLP in this pedigree (Table II). The greatest support is obtained when all three lipid measurements were analyzed jointly; a LOD score of 7.56 for a recombination fraction of zero was then obtained. The odds of this observation arising by chance alone are < 10⁻⁷.

Figure 1. Abridged presentation of pedigree 635. Only the family branches that were ascertained through field survey are represented. Haplotype 1 consists of allele 1 with Xba I and allele 2 with Msp I. In some individuals, the familial segregation of the Xba I and Msp I polymorphisms did not permit unambiguous inference of haplotypes. When lipid values did not allow classification into HBLP with sufficient confidence, subjects were referred to as borderline. When the chromosomal distribution of the apo B Msp I and Xba I alleles could not be determined from the pedigree data, these individuals were identified by the designation "haplotype not inferred."

Table I. Individual Data on Pedigree 635

Id	Sex	Age	CH	TG	VLDL	LDL	HDL	apo B	E	Msp I	Xba I
7	F	93	75 L	69	10	19 L	46	27	3 3	1 2	1 1
10	F	83	264	114	29	189	46	175	3 3	1 2	1 2
12	F	80	164 L	378 H	64	66 L	34	94	4 3	NA	1 2
13	F	76	232	213	95	95 L	42	NA	2 2	2 2	2 2
15	F	75	103 L	145	21	37 L	45	NA	3 2	2 2	1 2
16	M	71	82 L	49 L	2	39 L	41	NA	3 2	2 2	1 2
19	F	67	107 L	159	35	36 L	36	NA	3 2	2 2	1 2
20	F	64	227	242 H	122	59 L	46	NA	2 2	2 2	2 2
21	F	63	220	126	32	138	50	NA	3 2	2 2	2 2
22	M	71	108 L	56 L	14	46 L	48	38	3 3	2 3	1 1
23	F	68	268	166	74	157	37	171	4 3	1 3	1 1
24	F	67	134 L	85	12	37 L	85	31	4 3	2 3	1 1
25	M	61	91 L	98	7	45 L	39	37	3 3	2 3	1 1
26	M	63	160	226	45	87	28 L	116	4 3	1 2	1 2
28	F	58	149 L	44 L	22	41 L	86	39	4 4	2 2	1 2
29	F	34	135	67	20	60 L	53	54	4 3	2 2	1 2
30	F	62	341 H	241 H	60	242 H	39	175	3 2	2 2	2 2
31	M	53	293 H	148	38	217 H	38	NA	3 2	2 2	2 2
32	F	47	167	95	12	109	46	75	3 3	2 2	1 2
33	M	42	174	95	8	119	43	90	3 3	1 2	1 2
34	F	42	80 L	35 L	3	19 L	58	18	3 2	2 2	1 2
35	M	60	231	111	41	158	32	139	3 2	NA	1 2
36	F	59	123 L	68	14	56 L	53	38	4 2	2 2	1 2
37	F	57	248	59	14	178	56	154	3 3	1 2	1 2
38	M	53	130 L	101	17	74 L	39	77	3 3	1 1	1 1
39	F	41	167	90	14	101	52	84	3 3	NA	1 1
40	F	47	195	151	35	98	62	105	3 3	2 2	2 2
41	M	43	101 L	48 L	8	56 L	37	41	3 3	2 2	1 2
42	F	40	238	162	46	159	33 L	173	3 3	2 2	2 2
43	F	40	182	134	18	116	48	98	3 2	2 2	1 2
44	M	49	225	609 H	75	125	25 L	136	3 2	2 2	2 2
45	F	45	87 L	43 L	15	40 L	32 L	36	3 3	2 2	1 2
46	M	19	188	168 H	31	119	38	107	3 3	1 2	1 2
47	F	18	82 L	32 L	9	20 L	53	22	3 2	2 2	1 2
48	M	17	109 L	37 L	7	22 L	80 H	20	3 2	1 2	1 1
49	F	14	165	113	9	105	41	85	3 3	2 2	2 2
50	M	13	184	110	20	109	55	94	3 2	2 2	2 2
51	F	10	95 L	53	6	36 L	53	34	3 3	1 2	1 1
52	F	7	167	97	25	101	41	98	3 3	1 2	1 2

Total cholesterol (CH), triglycerides (TG), cholesterol measurements in lipoprotein fractions (VLDL, LDL, and HDL) and apo B quantitation (apo B) are expressed in milligrams per deciliter. E stands for the apo E phenotype determined by isoelectric focusing; Msp I and Xba I are two genotypes at the apo B locus. NA, not available. When LRC reference values are available, values below the 5th (L) and above the 95th percentile (H) are flagged.

To summarize the segregation and linkage evidence in a simple and descriptive manner, the estimated means and variance-covariances of the three lipid measurements were used to create a linear combination within each genotypic class. This procedure led to the definition of a compound variable for each individual assayed in the pedigree. For ease of presentation, all measurements were centered to the midpoint of the interval defined by the expected means of this compound in each genotypic class. Again, for descriptive purposes only, individuals could then be classified as HBLP or normal subjects, depending on whether they had or had not inherited the apo B

haplotype that segregates with HBLP. The linkage evidence thus can be described approximately as reflected in Fig. 1. The expected distribution and the observed values for this linear combination are reported in Fig. 2. Note that although the cosegregation of HBLP and the apo B marker is clearly apparent, a few individuals seem not to have inherited HBLP jointly with the apo B marker. This indicates that unambiguous identification of heterozygotes cannot be achieved on the basis of these measurements alone. Analyzing the quantitative values rather than making a dichotomic classification before the analysis circumvented the issue of genotypic identification.

Table II. Segregation and Linkage Analysis of Hypobetalipoproteinemia in Pedigree 635

Variable(s)	Genotypic means		SD	Recombination	LOD score
	Normal	Carrier			
(A) CH	198.76	101.16	34.62	0.0	6.96
(B) LDL	133.13	38.22	36.62	0.0	4.93
(C) TG	135.50	52.46	40.50	0.0	2.21
(D) apo B	121.62	38.26	28.32	0.0	4.48
(E) CH	190.05	96.33	31.66	0.0	7.56
TG	4.91	3.93	0.49		
LDL	118.14	36.68	34.63		

CH, total cholesterol; TG, triglycerides levels; LDL, LDL cholesterol; SD, standard deviation within genotypic distribution. CH alone was used in model A, whereas all three measurements were used in model B. Genotypic means, variance-covariances, and recombination rate between a postulated HBLP locus and apo B were jointly estimated. Logarithmic transformation was applied to triglycerides in the analysis.

Discussion

Cosegregation of hypobetalipoproteinemia with a particular allele of the apo B gene was demonstrated in a pedigree with a dominant pattern of inheritance of low LDL cholesterol levels. A defect of the apo B gene is probably responsible for the

occurrence of the lipid disorder in this pedigree. In a recent study, individuals in one family with HBLP were found to have low amounts of a small apo B protein, called B-37, in their plasma. This is presumably due to a mutation in the apo B gene that results in a truncated, unstable, or poorly synthesized protein (22, 23). In an investigation of two homozygous HBLP patients, it was shown that apo B mRNA levels and apo B levels were sharply reduced in liver cells (24). The results of these studies are compatible with the notion that mutations in the apo B gene cause HBLP.

17 of the 39 sampled individuals carried the apo B haplotype that segregates with HBLP. One of these carriers, subject 32, exhibited almost normal lipid levels: total cholesterol of 167 mg/dl, LDL cholesterol levels of 109 mg/dl, triglyceride levels of 95 mg/dl, and apo B concentrations of 75 mg/dl. This 47-yr-old woman is the sister of our initial proband, subject 34. Two other siblings, subjects 30 and 31, and the mother of those two siblings, subject 10, had high levels of total cholesterol or LDL cholesterol. Indeed, a pedigree branch of relatives of subject 10, with several cases of familial hypercholesterolemia and premature myocardial infarction, had led to the ascertainment of the HBLP proband, subject 34. Subject 32 conceivably carries two mutations at either one locus or at two different loci, each exerting an opposite effect on cholesterol metabolism.

One person who did not carry the HBLP haplotype, subject 38, had lipid values similar to those obtained in the mildest case of HBLP, subject 29. Unambiguous classification of HBLP based solely on lipid profiles, or on LDL cholesterol alone, is therefore not possible in this pedigree. Furthermore, subject 20, who does not carry the HBLP haplotype, had a total cholesterol level of 227 mg/dl, a triglyceride level of 242 mg/dl, a VLDL cholesterol level of 122 mg/dl, an LDL cholesterol level of 59 mg/dl, and an HDL cholesterol level of 46 mg/dl. This subject presents the E2/2 phenotype at the apo E locus, and his lipid profile is characteristic of type III hyperlipoproteinemia. Defective apo E is thought to result in low LDL cholesterol levels by inhibiting the normal progression from VLDL to intermediate density lipoproteins and to LDL, and/or by failing to deliver enough intermediate density lipoproteins and chylomicron remnants to the liver to down-regulate hepatic LDL receptors (25). The resultant up-regulation of LDL receptors is thought to cause a decrease in plasma LDL cholesterol levels. Various common genetic variants and environmental determinants that affect lipid levels in defined pedigrees, constitute a difficult challenge for genetic investigators.

In summary, through linkage analysis, we have presented strong evidence that a defect in the apo B gene is responsible for the occurrence of HBLP in an Idaho pedigree. The actual mutation involved remains to be identified. Our study also illustrates the power of the candidate gene approach for investigating the etiology of complex disorders of metabolism.

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Figure 2. The expected distribution of a linear combination of total cholesterol, the natural logarithm of triglycerides, and LDL-cholesterol based on the estimates of Table II, model E. Genotypic distributions were standardized to unit variance and the scale was centered to the midpoint of the interval between genotypic means. The same transformation was applied to the individual data of Table I, and the individual scores are reported in the figure. The lower bar diagram documents the values of this index among individuals carrying the apo B haplotype that segregates with HBLP; the upper diagram reports the corresponding values among individuals who did not exhibit this haplotype. The highest value among carriers was found in individual 32; the lowest value among subjects not carrying the haplotype was found in individual 38. Both would have been misclassified if a cut-off point had been defined before the analysis.

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